# The Journal of Comparative Pathology and Therapeutics

Vol. 68. No. 4 Price Seventeen Shillings and Sixpence

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# THE INTRAOCULAR LESIONS OF HOG CHOLERA

By

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## INTRODUCTION

In a study of dogs affected with canine distemper, Jubb, Saunders and Coates (1957) found that the virus invaded the retina and elicited degenerative and inflammatory changes in it.

Amongst naturally occurring viral infections which cause encephalitis in domestic animals, retinitis had also been described in Borna encephalitis of horses (Gmelin, 1931; Stofer, 1934; Walther, 1952), a disease in which clinical signs of blindness or impaired pupillary reflexes are often seen (Müller and Fritzsch, 1955). In addition, Le Vasseur in 1911 described Negri bodies in the retina of rabid dogs and rabbits in a report which had been forgotten by subsequent authors and indeed had been overlooked by ourselves in our previous paper (Jubb *et al.*, 1957).

In an attempt to determine whether invasion of the intraocular tissues is an attribute of other viruses which cause encephalitis we extended our observations to hog cholera. Although blindness is not usually seen in hog cholera, a histologic investigation of the eyes seemed worth while, since Seifried found that many more pigs had nervous lesions than had nervous symptoms in this disease. The histopathology of hog cholera—including its neuropathology—has been described in considerable detail (Hemboldt and Jungherr, 1952; Potel, 1956; Röhrer, 1930; Seifried, 1931), but previous investigators have not extended their observations to the intraocular tissues. De Kock, Robinson and Keppel (1940) mentioned blindness accompanied by atrophy of the bulb in chronic African swine fever, but did not refer to histologic changes.

## MATERIALS AND METHODS

Eye sections were studied from four naturally occurring cases of the acute disease; mild inflammatory changes were present in two of them. The use of field cases of hog cholera in a scientific study is subject to adverse criticism unless the virus is isolated by pig inoculation from each case. Even then it is impossible at times to separate the effects of the virus from those of secondary bacterial infections which may accompany it. For this reason, the major portion of this study was conducted with material from experimentally infected swine.

The eyes from 31 swine were used in the experimental phase of this study and those from 4 healthy pigs were available as controls. One eye,

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taken from each of 27 pigs which had been injected with hog cholera virus and killed 6 days later in the course of commercial virus production, was fixed immediately after enucleation in formol-saline. The exigencies of commercial virus production dictated that the swine involved be killed 6 days after inoculation. Mild lesions were seen histologically in 16 of the 27 swine. These findings, together with those in the natural cases, indicated that additional observations should be made on artificially infected animals that were allowed to live more than 6 days following inoculation.

On two separate occasions, two healthy young pigs from two unvaccinated herds were isolated in the laboratory on a clean concrete floor bedded with straw. The bedding was changed daily to simulate the strict sanitary environment of the commercial virus laboratory. One of the pigs was injected subcutaneously with 1 ml of commercial hog cholera virus and killed with pentobarbital sodium 9 days later. The second one was kept in pen contact with the first and similarly killed 14 days later. Both pigs developed a febrile illness with anorexia and posterior incoordination; conjunctivitis or visual disturbance was not seen. The eyes were enucleated immediately after death and fixed in Zenker-acetic; the other tissues were fixed in aqueous formalin. At autopsy both had typical gross lesions of hog cholera in the lymph nodes and viscera, and histologically also non-suppurative encephalomyelitis.

The third and fourth pigs were both inoculated with 1 ml of the same virus subcutaneously. They also developed clinical signs of acute hog cholera, but in this instance they were not killed. Mild mucous conjunctivitis developed in these animals about 2 days ante mortem. One died 11 days and the other 13 days after inoculation; post mortem examination was made within 15 minutes and 1 hour respectively. Gross lesions characteristic of hog cholera were present, and a non-suppurative encephalomyelitis was demonstrated histologically. No lesions of swine erysipelas or necrotic enteritis were seen in these four animals. The eyes and other tissues were fixed as in the first two pigs.

Because of an old report in the German literature that inclusion bodies occurred in the conjunctival epithelium in hog cholera, conjunctival tissue was cut with scissors from the latter four pigs and impression smears made and stained with Giemsa's stain. Inclusion bodies were not seen. Unfortunately, technical considerations precluded examination of the brain and spinal cord of all but the last four experimentally infected pigs. The lateral calottes were removed from all of the fixed eyes and embedded in paraffin; the remainder of the eye with the optic nerve was embedded in celloidin. Both types of sections were stained with haematoxylin and eosin, and additional sections from some cases were stained with cresyl violet, Mallory's trichrome and Weil's myelin sheath stain.

## RESULTS

No gross lesions were seen during the removal of the calottes. Histologically, inflammatory lesions were found in the retina and in all three portions of the uveal tract. The source of the animals studied and the distribution of the lesions are shown in Table 1. Extensive and well-marked lesions were only seen in the last 4 animals, and particularly in the two that were allowed to die. No histologic changes were seen in the controls.

TABLE 1

DISTRIBUTION OF INTRAOCULAR LESIONS IN 34 CASES OF HOG CHOLERA

| | *Number* | *No lesions* | *Optic nerve* | *Retina* | *Choroid* | *Ciliary body* | *Iris* |
|---|---|---|---|---|---|---|---|
| Field cases | 4 | 2 | 1 | 2 | 2 | — | 1 |
| Experimental A | 27 | 13 | 6 | 12 | 4 | 5 | 6 |
| Experimental B | 4 | — | 2 | 3 | 1 | 4 | 4 |

Experimental group A from commercial virus laboratory, killed 6 days following inoculation.

Accumulations of mononuclear cells were present in the iris. Some were identifiable as plasma cells, but the majority must be non-commitally designated as above. These cell clusters were seen in the middle of the iris (Fig. 1) as well as near the margins. The ciliary body was infiltrated by mononuclear cells which extended some distance into the processes (Fig. 2). Some of these appeared to be endothelial cells derived from the vessel walls in the processes; where they appeared at all they were heaped thickly enough to make recognition difficult. In a few cases the ciliary processes contained oedema and haemorrhage rather than mononuclear cells. Perivascular cuffing of blood vessels, usually veins, occurred in the retina (Fig. 3). The cuffs were made up of large mononuclear cells with a large, darkly staining rounded nucleus and a scanty cytoplasm. Proliferation of glial cells was occasionally seen in the retina, both in proximity to perivascular cuffing (Fig. 3) and as an independent lesion (Fig. 4). Proliferation of vascular endothelium was seen in the iris, choroid and optic nerve, but most clearly in the retina (Figs. 5 and 6). Optic neuritis, characterized chiefly by proliferation of glial cells, was well marked in 3 cases (Figs. 7, 8). There was no destruction of myelin in the optic nerve as is seen in distemper. No lesions were detected in the corneal endothelium, rods and cones, or retinal pigment epithelium. Focal mononuclear cell infiltration was present in the choroid in 3 cases which also had cuffing of the retinal vessels, and in four in which only anterior uveal lesions were present. The choroidal infiltration was the mildest and least extensive of the changes seen. Ganglion cell degeneration was likewise an occasional feature and was recognized unequivocally only in two cases.

## DISCUSSION

It appears from these observations that the virus invades both the retina and uveal tract, but that significant changes do not occur much before 6 days following infection and are best seen in animals infected 9 days or longer. Some of the lesions seem to indicate the hematogenous arrival of the virus in the eye and its attack on the

endothelium of the intraocular blood vessels. We know of but one experimental study in which inflammatory intraocular lesions were reported in swine. Fog (1952) found encephalitis and iridocyclitis in young pigs that he injected and later re-injected with brain tissue from human multiple sclerosis. Since there is no widespread myelin destruction in hog cholera encephalitis, this allergic mechanism cannot be invoked to explain the intraocular lesions in our animals.

The sparing of the retinal ganglion cells is at variance with our findings in canine distemper. Since there was no demonstrable fibre destruction in the optic nerves, this sparing may be due to the fact that the ganglion cells did not have their axons severed as so often occurs in canine distemper. The lack of reaction on the part of the retinal pigment epithelium was somewhat surprising; apparently that of the pig does not react to injury as readily as does that of the dog. The perivascular cuffing in the retina was milder than that seen in some of our canine distemper cases. This may be related to the fact that the encephalomyelitis, in the four cases where we were able to study the nervous system, was likewise less severe and extensive than we have on other occasions encountered in field cases of hog cholera. As the latter were studied some years ago, at a time when we had no facilities for eye sectioning, we can only speculate whether a more severe retinitis would have been found in those cases.

Although we saw occasional occlusion of capillaries in the choroid and iris by proliferating endothelium, we found no lesions of infarction, such as occurred in the spleens of two of our pigs and might have been expected to occur in other organs. The accumulation of foci of mononuclear cells in the iris and ciliary body is a change we have seen elsewhere only in periodic ophthalmia of horses. The chorioretinal changes are not unlike those described in Borna encephalitis in which disease, however, the neural elements of the retina are also damaged.

The hog cholera virus, like that of canine distemper, is a pantropic agent localizing in and damaging many tissues of the body. Some of the damage that it wreaks in the eye is not unlike that occasioned in the central nervous system of the same animals. While this is of interest in extending our knowledge of this virus, it is of even greater interest because of the common pattern that is apparently emerging. Here are 4 viral agents, rabies, distemper, Borna encephalitis and hog cholera, which cause encephalitis and also concurrently cause degenerative or inflammatory changes in the eye, especially in the retina. If this pattern holds true in the case of several other viruses, it may well transpire that intraocular damage is a general characteristic of such agents.

## CONCLUSIONS

Intraocular inflammatory changes were found in a histologic study of natural and experimental cases of hog cholera. The lesions were chiefly retinitis and anterior uveitis; in a few cases choroiditis

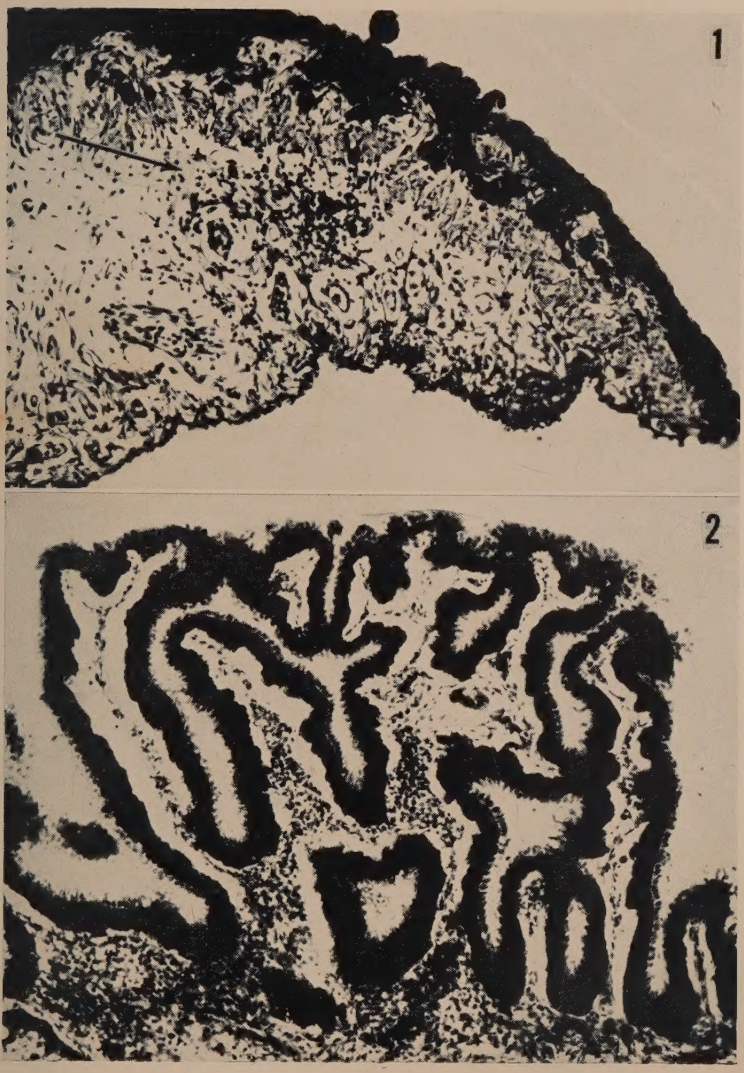


Fig. 1. Accumulation of mononuclear inflammatory cells in the iris. This and all subsequent figures, from sections stained with H. & E. × 80.

Fig. 2. Infiltration of the ciliary body by mononuclear leucocytes and proliferating endothelial cells. × 80.

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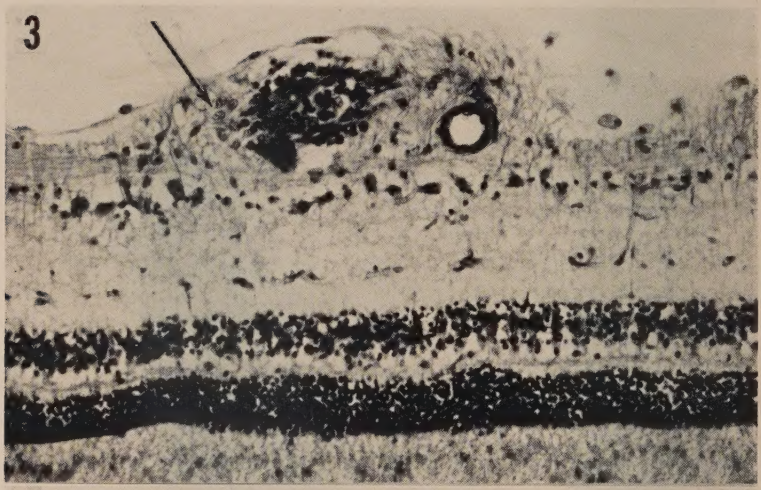


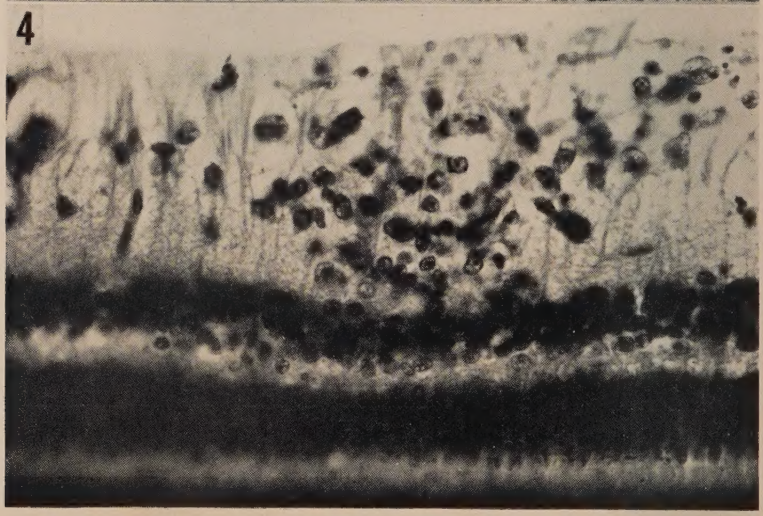


Fig. 3. Perivascular cuffing of a vein in the optic fibre layer of the retina. The arrow points to an adjacent accumulation of glial cells. × 200.

Fig. 4. Accumulation of glial cells in the ganglion cell and inner reticular layers of the retina. × 320.

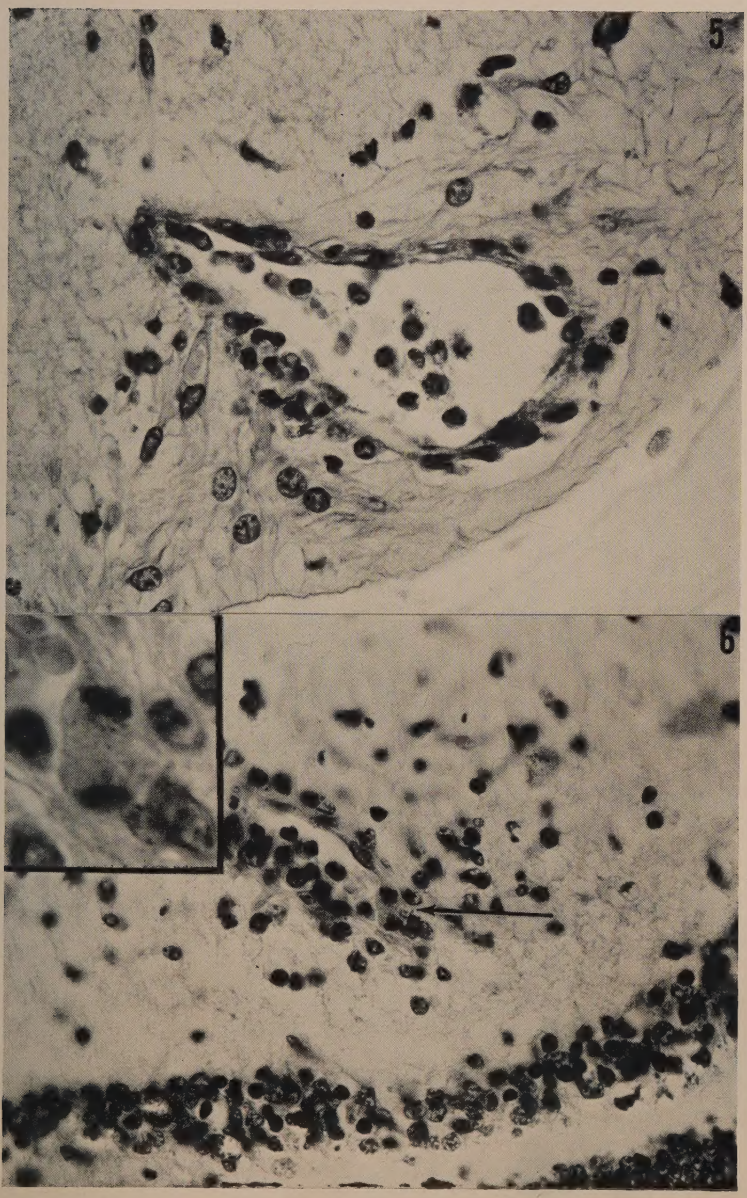


Fig. 5. Vein in oedematous optic fibre layer of the retina with an area of proliferating endothelial cells. × 320.

Fig. 6. Vein in ganglion cell layer of the retina with beginning perivascular cuffing and proliferation of endothelium. The arrow points to an endothelial cell undergoing mitosis, × 320; a higher magnification of this cell appears in the inset, × 900.

Fig. 7. Gliosis of the optic nerve and optic disc, perivascular glial and leucocytic infiltration of the central vessels in the disc. × 38.

Fig. 8. Longitudinal section of optic nerve showing patchy distribution of gliosis, × 80. The inset shows a group of these glial cells, with one in mitosis, × 330.

was present as well. These findings, which are analogous to the lesions occurring in canine distemper and Borna encephalitis, suggest that invasion of the intraocular tissues is a fundamental attribute of viruses which cause encephalomyelitis in animals.

ACKNOWLEDGMENTS

We are indebted to Mr. R. F. Smith for the photomicrographs. Part of this study was carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

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[*Received for publication, November 1st,* 1957]

# STUDIES ON THE HETEROPHILE ANTIBODIES ASSOCIATED WITH HELMINTH INFECTIONS

## III. Heterophile Antibody in *Oesophagostomum dentatum* Infection in Pigs

By

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### INTRODUCTION

In a previous communication (Soulsby, 1958) it was reported that pigs infected with *Ascaris lumbricoides* showed a marked elevation of heterophile antibody titres, which at times reached extremely high levels. It was suggested that this antibody was a "low molecular" type of Forssman antibody, possessing little or no haemolytic ability for sheep erythrocytes, and corresponded to a low molecular weight Forssman antibody described by Talmage and Freter (1956). Absorption of sera with larvae of *A. lumbricoides* removed this antibody and absorption also occurred with *Oesophagostomum dentatum* larvae indicating that Oesophagostomum infection in pigs might also play a part in modifying heterophile antibody levels. This communication deals with a study of the serum and faecal heterophile antibody levels in a group of pigs experimentally infected with *O. dentatum*. Since later in the experiment the pigs became infected with intercurrent bacterial disease the work is also to some extent an examination of heterophile antibodies occurring in pigs under natural conditions of infection by pig pathogens.

### MATERIALS AND METHODS

*Animals.* A group of five pigs were obtained by Caesarian section and were raised in sterilized cages on an artificial diet, being deprived of colostrum. During the experiment they were kept in thoroughly cleansed pens and were infected with *O. dentatum* larvae at 7 weeks of age. Blood samples were taken from ear veins and serum was collected after overnight separation. Faeces samples were taken from the rectum; part of the sample was used for egg counts and the remainder was added to four times its weight of saline, formalin being added to give a final concentration of 0·5 per cent (Burrows, Elliott and Havens, 1947). After thorough mixing the treated faeces sample was centrifuged at 4,000 r.p.m. for 30 minutes. The supernatant was removed and constituted the preparation for the determination of faecal antibodies. It was regarded as undiluted material in serological tests, but is referred to as a 1 in 4 dilution in the presentation of the results. Blood serum and faecal preparations were stored at less than −20°C and were inactivated at 56°C for 30 minutes before use.

*Parasite materials. O. dentatum* third stage larvae were obtained from sterile peat moss cultures of pig faeces showing high egg counts for *O.*

*dentatum*. Separation of larvae was by the Baermann technique and larvae were administered to the pigs orally.

*Serological tests*. Serum and faecal samples were tested for heterophile antibodies by both a haemolytic test and an "interrupted" conglutination test as previously described (Soulsby, 1958).

## RESULTS

Three pigs (1, 2 and 3) were infected with 150,000 third stage *O. dentatum* larvae and a further two (4 and 5) served as uninfected controls. The resulting serum and faecal heterophile antibody levels, as determined by the conglutination test, are illustrated in Figs. 1 and 2. Patency of the oesophagostomum infection occurred in pigs 1, 2 and 3 on days 35, 31 and 49 after infection respectively.

Whilst the three infected pigs showed a substantial rise in heterophile antibody after infection a similar marked rise occurred in one control pig (5). It is noteworthy that the serum heterophile antibody levels showed very marked variation throughout the period of the experiment. In two infected pigs (1 and 3) patency of the helminth infection appeared to be associated with an increase of heterophile antibody in the faeces and to some extent in the serum, but the significance of this will be discussed later. With a few exceptions the titres of heterophile antibody as detected by a haemolytic test were uniformly negative. If, however, an interrupted test was carried out to avoid the anticomplementary action of pig serum on guinea pig complement then haemolytic titres could be raised, but even so they were still of a very low order.

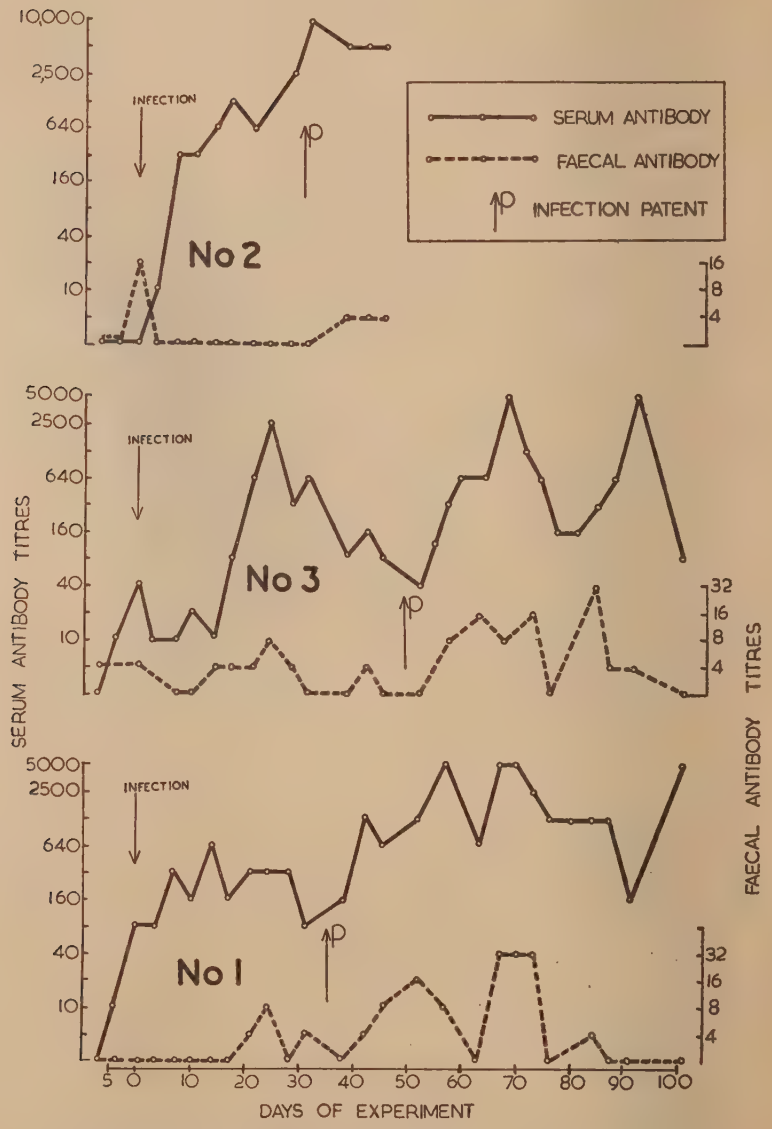
From the period of 40 days onwards all the pigs suffered from swine erysipelas and were treated with penicillin when clinical symptoms became evident.

### *Identity of the Antibody*

The faecal antibody titres were too low to carry out any detailed examination, but antibody in the faecal material could be absorbed completely by the use of sheep red blood cells, ground up guinea pig kidney and fowl red blood cells, thus indicating that it was heterophile in nature. The serum antibody was subjected to a closer examination. Sheep erythrocytes completely absorbed the antibody from the sera of infected and control animals. None of the sera from infected pigs agglutinated fowl (hen) erythrocytes and generally the sera from the control pigs were negative, although an occasional serum sample agglutinated these cells to a titre of 1 in 10 when the sheep red cell conglutination titre was 1 in 10,000 (Pig 5). Sheep erythrocytes were not agglutinated, thus indicating that the heterophile complex lacked the Paul Bunnel type of antibody.

Further examination of sera from infected and control pigs early and late in the experiment revealed differences in heterophile specificity, but it soon became obvious that a complexity of heterophile antibodies was present and no definite decision could be

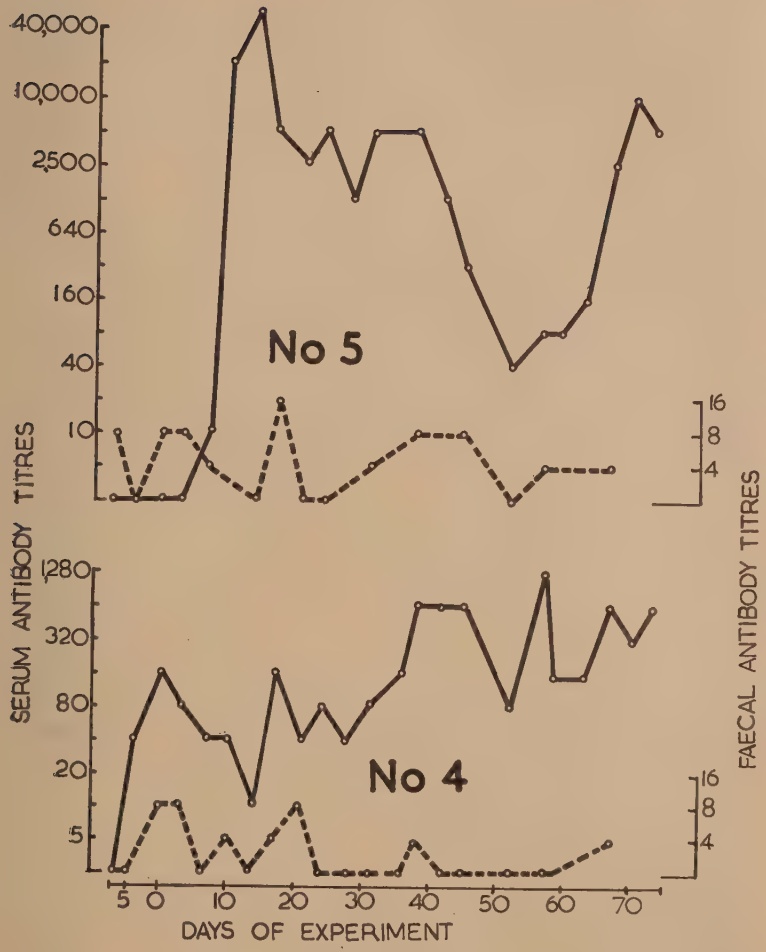
Fig. 1




Serum and faecal heterophile antibody levels in three pigs each infected with 150,000 third stage larvae of *Oesophagostomum dentatum*. Antibody was detected by the conglutination test.

reached as to the part played by infectious agents in the stimulation of the complex. However, some general trends were present. The

Fig. 2




Serum and faecal heterophile antibody levels in two pigs not infected with *O. dentatum*. Antibody was detected by the conglutination test. Key to types of antibody as in Fig. 1.

heterophile antibody in control pigs early in the experiment was more readily absorbed by fowl erythrocytes than by guinea pig kidney, whilst that in the infected pigs early in the experiment proved to be more easily absorbable by guinea pig kidney preparations than by fowl erythrocytes. In each case the residue of antibody after absorption with one material was removed by absorption with the second. The antibody in both infected and control pigs late

in the experiment showed greater absorbability with fowl erythrocytes than with guinea pig kidney. In fact, in one pig (No. 3) some serum samples late in the experiment showed complete refractoriness to absorption with guinea pig kidney. It would appear, therefore, that in pig serum a heterophile antibody complex occurs which has two main specificities, one for fowl erythrocytes and one for guinea pig kidney and in the case of oesophagostomum infection the guinea pig kidney specificity would appear to predominate in the early stages of infection.

Agglutinins for pigeon erythrocytes were present in sera from these pigs. In control pigs, pigeon erythrocyte agglutinins were present in substantial titre both early and late in the experiment, with a maximum titre of 1 in 5,000 in the case of pig 5 early in the experiment. In infected pigs, even at the peak titres for the conglutination of sheep erythrocytes, pigeon erythrocyte agglutinins did not exceed a titre of 1 in 40. Here again the specificity of the heterophile antibody complex early in the experiment showed a difference between infected and control pigs in that controls had much bigger titres of pigeon erythrocyte agglutinins. This indicates, at least, that oesophagostomum infection in pigs is not associated with the marked production of pigeon erythrocyte agglutinins. The pigeon erythrocyte agglutinins were more readily absorbed by guinea pig kidney than by fowl erythrocytes and the residue of agglutinin from absorption with one material could be removed by absorption with the other material.

Since the occurrence of pigeon erythrocyte agglutinins is indicative of the haemorrhagic septicaemia—bird erythrocyte group of heterophile antibodies (Buchbinder, 1935), sera were absorbed with Pasteurella organisms. Double absorption with such organisms resulted in a lowering of conglutination titres for sheep erythrocytes, but though relatively efficient, Pasteurella organisms were unable to remove completely heterophile antibodies and a substantial legacy of heterophile activity remained after repeated absorptions. There was, however, a reasonably efficient removal of pigeon erythrocyte agglutinins by Pasteurella organisms, only a small amount of agglutinin activity being non-absorbable. Absorption of sera with *Erysipelothrix rhusiopathiae* organisms removed to some extent heterophile antibodies in both control and infected pigs, absorption being more efficient in the sera from control pigs.

## DISCUSSION

The first point concerning the heterophile antibody levels in pigs is that a marked variation can be expected even in pigs which have not received any experimental infections. Whilst there is evidence of the presence of the Forssman antigen in the third stage larvae of *O. dentatum* (Soulsby, 1958) the present experiments give no unequivocal evidence that heterophile antibody titres are markedly

elevated on infection with third stage larvae. There does appear to be a stimulation of heterophile antibody levels following patency of the oesophagostomum infection and in two pigs this is coupled with an increase in the amount of antibody detectable in the faeces.

From absorption tests it would appear that the heterophile antibody during the early stages of *Oesophagostomum* infection is principally of guinea pig kidney specificity as compared with the fowl erythrocyte specificity of the antibody seen in the control pigs at this stage. Following patency of the oesophagostomum infection, however, the specificity of the antibody in infected pigs tends to change to that of fowl erythrocytes, in other words to resemble that in the controls. Since at this period all pigs underwent a natural attack of swine erysipelas it is possible that this infection and organisms associated with it induced the latter type of heterophile antibody in both controls and infected pigs and that the rises in heterophile antibody at the time of patency of the helminth infection are fortuitously due to bacterial infection.

Another difference between the antibody in control and infected pigs is the ability to agglutinate pigeon erythrocytes, this being much more marked in the control pigs. This indicates that *O. dentatum* infection is not associated with the marked stimulation of such agglutinins. Since pigeon erythrocyte agglutinins could be fairly efficiently absorbed by Pasteurella organisms it is reasonable to assume that these organisms were probably responsible for their stimulation and furthermore may well have been responsible for the high levels of heterophile antibody as determined by the conglutination test in control pig 5 since such antibodies were partially absorbable by pasteurella organisms. Erysipelothrix organisms also appear to play a part in the stimulation of heterophile antibodies.

The broad picture of the results in these pigs is that a marked variation in heterophile antibody levels occurs under the influence of natural infectious agents. The heterophile antibodies can be detected by the use of sheep erythrocytes as an indicator and are absorbed by sheep erythrocytes. But within this general picture a fairly complex heterophile specificity exists. In order to clarify the part played by each infectious agent in the stimulation of a particular heterophile antibody it will be necessary to carry out further work using animals infected with a single infectious agent. This report merely serves to indicate that a complex of heterophile antibodies occurs in the blood of pigs.

The levels of serum heterophile antibody are of further interest in that previous to infection the titres are either very low or negative both in control and infected pigs. At the time of infection, i.e. 7 weeks of age, heterophile antibodies were produced in substantial amounts irrespective of infection. It is doubtful whether these pre-infection titres, despite their low nature, can be regarded as "natural" or "normal" antibody and they probably represent the initiation of an antibody response to the natural pathogens of pigs. The negative

periods previous to this are probably indicative of the inability of pigs to produce demonstrable antibody. Since these pigs were deprived of colostrum they resemble those used by Hoerlein (1957) who found that such pigs did not develop antibodies to injected antigens previous to 8 weeks of age. Whilst the experiments of Hoerlein were concerned with the artificial introduction of antigenic materials into colostrum-deprived pigs, those reported here concern the response of the pig to infections which it may fortuitously encounter in life. If the inability to show an antibody response previous to 6 or 7 weeks of age is a measure of the ability to become immune to infections then it is obvious that colostrum-deprived pigs of such an age are highly susceptible to many infectious agents other than parasitic worms.

The presence of heterophile antibodies in the faeces raises questions as to the origin of such materials. Burrows, Deupree and Moore (1950) have discussed the evidence for and against local production in the bowel as opposed to diffusion of antibody through the bowel wall from the plasma. It is obvious that faecal antibodies can never be expected to reach the levels which are found in the serum since no accumulation of antibody is allowed to occur, as it is being constantly eliminated in the faeces. Any high level of faecal antibody is, therefore, a measure of either the amount being produced locally in the bowel wall or diffusing from the blood. In the pigs in question, as a rule, high levels of faecal antibody either coincided with or shortly followed a high level of serum antibody and it would appear that in the majority of instances this antibody was derived from serum antibody. There are, however, two periods of faecal antibody output which are of interest. One occurs at the time of patency of the oesophagostomum infection. In pigs 1 and 2 the faecal antibody levels are more marked than in control pigs. Though much of this may be contributed by the existing high levels of serum antibody at that time it is possible that local production in the bowel wall may have been stimulated by the activity of the adult *O. dentatum* worms. That local production can occur in the absence of serum antibody is seen in the case of pigs 2, 3 and 5 when faecal antibody is present in the total absence of demonstrable serum antibody. In these pigs at least, the ability to produce heterophile antibody locally precedes the general process, but it would appear that the faecal antibody may be derived from both general and local sources.

## CONCLUSIONS

Heterophile antibodies of the Forssman type are stimulated in rabbits and pigs when infected with *Ascaris lumbricoides*: *Oesophagostomum dentatum* infection in the pig is also associated with an increase in such antibodies.

No protective mechanism can be ascribed to such antibodies, but in the rabbit the Forssman antibody induced by *A. lumbricoides*

infection can be used as a "marker" to detect slight changes in the antibody response which would not be detected by other techniques.

In pigs, the natural heterophile antibody titres undergo substantial variations. Infections with *A. lumbricoides* and with *O. dentatum* are thought to play a part in modifying these levels.

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[*Received for publication, December 6th,* 1957]

# STUDIES ON *ESCHERICHIA COLI* OF ANIMAL ORIGIN

## I. *E. coli* from Natural Outbreaks of Colibacillosis of Calves

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### INTRODUCTION

The continued association of coliform organisms with calf deaths in many parts of the world has prompted many workers to investigate the matter in some detail. Jensen (1891), working in Denmark, was the first to state that *E. coli* was a potential pathogen and since that date a large amount of work on the subject has been published which has been fully reviewed by Lovell (1955).

During the course of studies on young calves kept under experimental conditions at the National Institute for Research in Dairying, Shinfield between the years 1949–53, a number of pathogenic serotypes of *E. coli* were found (Ingram, Lovell, Wood, Aschaffenburg, Bartlett, Kon, Roy and Sears, 1953; Wood, 1955). From 1953 to 1956, while these studies were continued, a simultaneous investigation was made into naturally occurring outbreaks of white scours and colibacillosis of calves. Material from many parts of the country was provided by Veterinary Investigation Officers and veterinary surgeons in private practice who were asked to send cultures, samples of faeces, organs and anything which might contribute to this study. One hundred and thirty-one separate incidents were investigated. Some involved one animal only, in others the incidence of disease in many calves was studied over periods of up to six months. Data relevant to the cases discussed below are recorded in the Appendix and details of the remainder are given elsewhere (Rees, 1957a). The purpose of this paper is to discuss these data and relate them to a wider field of study.

### METHODS

The principal method of classifying strains of *E. coli* is based on the antigenic structure of this organism (Kauffmann, 1954). At present, there are at least one hundred and thirty-seven known somatic or 'O' antigens, eighty surface or 'K' antigens and forty-two flagellar or 'H' antigens. Not all strains however, are motile. The surface antigens may be divided into three types, L, A and B on the basis of their reaction to heat treatment.

*Antisera.* Most of the serotypes listed by Ingram *et al.* (1953) and Wood (1955) were used in the preparation of typing sera. To these were added a number of new and recurrent serotypes found in the course of these studies and nine others, normally associated with infantile gastro-enteritis. All the strains were isolated from calves.

*Calf strains.*

RVC51. O group 114 on the Kauffmann-Knipschildt-Vahlne Schema. Type B surface antigen.

RVC57. No known type.
RVC58. No known type.
RVC95. O group 26, B6.
RVC101. O group 35. Surface antigen not determined.
RVC118. O group 9. Type A surface antigen, different from that of RVC1798.
RVC237. No known type.
RVC330. O group 78. K 80.'(B).
RVC383. O group 15. Surface antigen not determined.
RVC608. No known type.
RVC924. O group 117. Surface antigen not determined.
RVC1034. No known type.
RVC1740. O group 24. Surface antigen not determined.
RVC1787. O group 137. K 79(L).
RVC1798. O group 9. Type A surface antigen different from that of RVC118.

Numbers prefixed by the letters RVC are the catalogue numbers of the strains isolated at the Royal Veterinary College and were given before their international antigenic formulae had been assigned to them.

*Strains from cases of infantile gastro-enteritis.* (O55.B5, O86.B7, O103.B?, O111.B4, O119.B14, O125.B15, O126.B16, O127.B8, O128.B12.) Care was taken in the selection of strains for antiserum production that highly motile organisms were not used. Though Kauffmann (1944) recommended the selection of non-motile or poorly motile strains he has said elsewhere (1954) that H agglutinins, even when present in antiserum, do not usually interfere in agglutination tests with twenty hour agar cultures. This is contrary to the findings of Wright and Villaneuva (1953, 1954) who have demonstrated such interference and advise workers to proceed with caution in this matter. Indeed, in the early part of this survey, some difficulties were experienced in the interpretation of results when very motile organisms had been used in antiserum preparation. Strains for inoculation were grown overnight on nutrient agar containing 0·1 per cent glucose which was incorporated because it promoted the development of K antigens (Kauffmann, 1947).

As both somatic and surface antigens have been associated with pathogenicity (Briggs, 1951; Briggs, Lovell, Aschaffenburg, Bartlett, Kon, Roy, Thompson and Walker, 1951; Kauffman, 1954; Smith, 1928; Smith and Bryant, 1927; Wramby, 1948), it was decided to search for complete strains rather than particular antigens.

*Slide agglutination.* Test sera were grouped together in pools of three or four for the preliminary sorting of strains. Those which were agglutinated by one of the serum pools were tested against its components.

*Tube agglutination.* Once a strain had been found to react with a particular serum, it was examined by tube agglutination to determine its somatic and surface antigens. Such strains were grown overnight on 0·1 per cent glucose agar and made up into saline suspensions with opacities equal to Brown's tube No. 1. The surface antigen was examined by testing the living suspension against serial dilutions of antiserum ranging from 1 : 40 to 1 : 2560 at a temperature of 37°C. Suspensions for somatic agglutination were heated before use (Kauffmann, 1954) and tested against serum dilutions of 1: 40 to 1: 10,240 at a temperature of

56°C. All agglutination tests were kept overnight at their respective temperatures before readings were taken.

## RESULTS

Most of the strains fermented lactose, produced indole, gave a positive methyl red test, did not produce acetylmethylcarbinol and failed to grow in Koser's citrate medium.

### *Strains from Outbreaks of Colibacillosis in Calves*

The total number of cases examined and the monthly incidence of serologically identifiable strains are given in Table 1. The monthly totals from this Table are compared in Table 2 with data abstracted from a report by Withers (1952). These are the monthly mortality rates for calves up to six months of age in British herds and are expressed as a percentage of live births. Deaths from all causes are included and his figures cover the years 1946–47–48. It will be seen that the peak death rate in Withers' findings occurs during March whereas the greatest influx of material during the 1953–6 survey occurs a month earlier. Other than this, there is agreement in so far as there is a steep rise in mortality throughout early winter, reaching a maximum in late winter and with a rapid decline in early spring.

Eleven different serotypes were recovered, some of them many times, the highest percentage of serotypes being found at the time of greatest mortality (Fig. 1). Total column height represents the number of cases examined during the corresponding months of three successive seasons.

The most frequently occurring type was O78,K80 which appeared on fifteen separate occasions. It was the only strain to appear in all three seasons and was the one most widely distributed: it was recovered from outbreaks in the north and south of Scotland, the south and west of England, Leicestershire and County Durham. Next in order of frequency came O137,K79 in seven outbreaks over two seasons. It was found widely in Scotland, but was also recovered from two cases in the south of England. The occurrence of serotypes O86,B7 and O103,B? hitherto associated with infantile gastro-enteritis has been recorded elsewhere (Rees, 1957b).

Where details were supplied, O35,K?, O78,K80 and O137,K79 were most often found to be associated with a virulent and rapidly fatal form of infection. On some occasions calves died before diarrhoea had appeared. The strains could be isolated in pure culture and in large numbers from all organs and there were often marked lesions of septicaemia. In outbreak No. 7, O137,K79 killed several one-day-old calves which showed weakness and scouring for only a few hours before death. Cultures from calves dying under identical circumstances on this farm six weeks later yielded the same serotype. The remaining serotypes were sometimes recovered from septicaemic animals, but more often the infection was localized to the intestinal

TABLE 1

THE INCIDENCE OF STRAINS OF *E. COLI* IN NATURALLY OCCURRING CASES OF COLIBACILLOSIS OF CALVES

| *Serotype* | *Sept.* | | | *Oct.* | | | *Nov.* | | | *Dec.* | | | *Jan.* | | | *Feb.* | | | *Mar.* | | | *Apr.* | | | *May* | | | *June* | | | *July* | | | *Aug.* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* |
| O9.A (type RVC118) | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | |
| O9.A (type RVC1798) | | | | | | | | | | | | 1 | | | | | | | | | 1 | | | | | 1 | | | | | | | | | | |
| O15.K? | | | | | | | | | | | 1 | | | | | | | | | | 1 | | | | | | | | | | | | | | | |
| O35.K? | | | | | | | | | | | | | | | 1 | | 1 | 1 | | | | | | 1 | | | | | | | | | | | | |
| O78.K80 | | | | | | | | | 2 | | 1 | | 2 | | 3 | | 1 | 4 | | | | | | 1 | | | | | | 1 | | | | | | |
| O86.B7 | | | | | | | | | | | | | | | | | | 1 | | | | | 1 | | | | | | | | | | | | | |
| O103.B? | | | | | | | | | | | | | | | 1 | | | 1 | | | | | | | | | | | | | | | | | | |
| O117.K? | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | | | |
| O137.K79 | | | | | | | | | | | | | | | | | 3 | | | 1 | 2 | | | | | 1 | | | | | | | | | | |
| RVC58 | | | | | | | | | | | | | | | | | | 1 | | | | | | | | 1 | | | | | | | | | | |
| RVC1034 | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| K. aerogenes | | | | | | | | | 1 | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | |
| Unidentified | | | | | | 1 | | 4 | 3 | 2 | 5 | 4 | 4 | 6 | 6 | 2 | 2 | 13 | 3 | 5 | 8 | 1 | 7 | 8 | | 1 | | | 1 | 1 | | | 1 | | | 1 | |
| Totals | — | — | — | — | — | 1 | — | 4 | 7 | 2 | 7 | 5 | 6 | 6 | 11 | 2 | 7 | 23 | 3 | 6 | 13 | 1 | 8 | 10 | — | 4 | — | — | 1 | 2 | — | — | 1 | — | 1 | — |
| | 0 | | | 1 | | | 11 | | | 14 | | | 23 | | | 32 | | | 22 | | | 19 | | | 4 | | | 3 | | | 1 | | | 1 | | |

COLUMN 1 in each month gives figures for the Season 1953–54, COLUMN 2 for 1954–55 and COLUMN 3 for 1955–56.

TABLE 2

| Data obtained from Withers (1952) | | | | Monthly totals from Table 1 |
|---|---|---|---|---|
| *Month* | *Live births* | *Number of deaths* | *Approx. % deaths* | |
| January | 785 | 49 | 6·2 | 23 |
| February | 871 | 65 | 7·5 | 32 |
| March | 714 | 75 | 8·3 | 22 |
| April | 647 | 52 | 8·0 | 19 |
| May | 469 | 33 | 6·4 | 4 |
| June | 419 | 20 | 4·8 | 3 |
| July | 395 | 18 | 4·7 | 1 |
| August | 535 | 14 | 2·7 | 1 |
| September | 937 | 25 | 2·7 | 0 |
| October | 1126 | 35 | 3·2 | 1 |
| November | 836 | 39 | 4·6 | 11 |
| December | 837 | 35 | 4·2 | 14 |

tract and associated lymph nodes. Scouring was the main sign and cultures from the liver and heart blood, when not sterile, gave very sparse growth compared with those taken from septicaemic animals. There were frequently no lesions of septicaemia, though a small number of infections seemed to correspond to a mild condition intermediate between a localized intestinal infection and a generalized septicaemia.

Despite the frequent lack of detailed information, sufficient data were supplied with some cases to give some indication of the epidemiology of colibacillosis. On five different farms (Nos. 7, 8, 12, 19 and 27) it was possible to associate particular types with outbreaks of colibacillosis lasting as long as six weeks. On another three (Nos. 2, 10 and 21), there was evidence of the transmission of such strains from one calf to another.

Sixteen histories recorded infection in calves even though they had been allowed to suckle or had been fed colostrum from a bucket. They were Nos. 1, 4, 5, 6, 8, 11, 13, 14, 16, 17, 18, 19, 20, 23, 25 and 26. Three of these were associated with O78,K80, one with O35,K? and one with O137,K79. Strains from the remainder could not be typed. On six farms (Nos. 3, 9, 15, 22, 23 and 24) where calves died of colibacillosis the farmers were in the habit of buying in new stock.

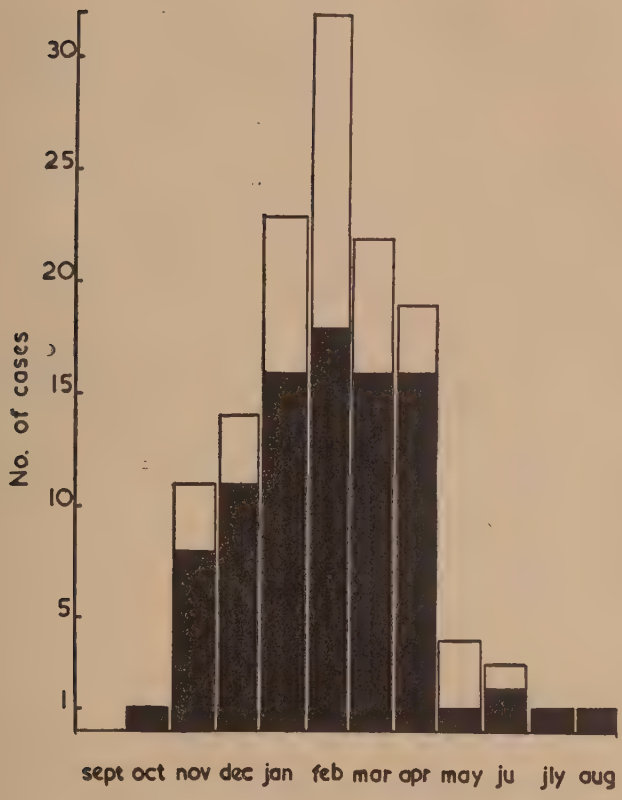


FIGURE 1

Monthly distribution of cases investigated during the seasons 1953–54, 1954–55 and 1955–56. Total column height represents total number investigated. Unshaded portion represents that number from which known serotypes were isolated.

On one occasion it was possible to obtain serum samples from cows in the same herd as a calf that died (No. 11). Serum from the dam contained no specific antibodies against the strain of *E. coli* which was recovered from her calf, but serum from another cow in the same herd agglutinated this strain to a titre of 1 in 160 in respect of its surface antigen.

E. coli *from the Udder Secretions of Cows with Mastitis*

During the course of the survey, coliform organisms isolated from a total of forty-six different samples of udder secretion were tested serologically against the antisera used currently in the work on

disease in calves. It was possible to identify the strains from four samples. Two were O9,A (type RVC1798), the third was O78,K80 and the fourth O117,K? Serological identities were confirmed by agglutinin cross-absorption tests with the corresponding type strains.

## DISCUSSION

Despite the existence of so many antigenic types of *E. coli*, a comparatively small number is regularly associated with colibacillosis of calves. These represent in natural outbreaks the recurrent serotypes found by Ingram *et al.* (1953) and Wood (1955) in experimental calves and correspond to the special races of Lovell (1937). Wood suggests that types such as these may undergo a process of natural selection and thus gradually became dominant in the flora of his community of experimental calves. This would presumably be linked with an increase in mortality. Roy, Palmer, Shillam, Ingram and Wood (1955) did, in fact, demonstrate such an increase which they related to the length of continuous occupation of the calf house. Figure 1 appears to reflect this process with regard to naturally occurring outbreaks of the disease in that the greatest percentage of serotypes was found during the period of highest mortality.

Individual serotypes, once introduced into a herd usually spread rapidly and may be shared by many animals within a short time. This undoubtedly holds for pathogenic and non-pathogenic strains and it may be assumed that in a herd of cattle the principal elements of the local coliform flora will be shared by adult animals and young alike.

At calving time, the colostrum of the dam would protect her new-born calf from infection by strains of *E. coli*, particularly if it contained specific antibodies against the surface antigens (Briggs *et al.*, 1951; Ingram, Lovell, Wood, Aschaffenburg, Bartlett, Kon, Palmer, Roy and Shillam, 1956). The antibodies most likely to be present would be those against strains present in the intestine of the dam herself; that is, some or all of the elements of the local coliform flora. In a case described above, the serum of a cow was devoid of antibodies against a strain which killed her calf. Another cow had these specific antibodies which were presumably present in her colostrum as well.

The coliform flora is dynamic however, and new strains may be introduced by a variety of means such as unhygienic practices or the introduction of new stock. If this happens during calving, newborn calves may be infected by them before there is time for specific antibodies to develop in the colostrum of their dams. When such young calves feed, they receive no protection beyond what non-specific effect the colostrum may possess. Should the new strain be a virulent type, an outbreak of white scours might be the consequence of its introduction. This is very possibly the explanation of the sixteen cases mentioned above, wherein calves are known to have died in

spite of their having received colostrum. It is noteworthy that five of these yielded known serotypes and were septicaemic. Of the three serotypes associated with these deaths, O78,K80 has also been found by Ingram *et al.* (1956) in colostrum-fed experimental calves though death in these latter animals was usually associated with a localized intestinal infection and not a septicaemia.

On six farms where calves became infected, the owners were in the habit of buying new stock. Two of these outbreaks were associated with O137,K79. On one of these two farms, the calves were given colostrum. No information was available about the feeding or withholding of colostrum on the other farms.

The number of serotypes of *E. coli* specially pathogenic for calves is unknown, but already there appear to be too many for the preparation of a comprehensive immune serum. It is interesting, however, that serotypes already incriminated in colibacillosis of calves should be recovered from the udder secretions of cows. Though nothing is known about the incidence of white scours on the farms from which the samples were obtained, it is possible to speculate on the nature of potential reservoirs of infection for young calves.

Those serotypes usually associated with white scours seem to occur most frequently in the bovine coliform flora and this is the most probable source of infection. Other animals on the farm may also pick up these strains and may easily transmit them to their original source at a later date. Three 'calf' serotypes have already been recovered from cases of enteritis in lambs (Rees, 1958).

## CONCLUSIONS

The findings of a survey into naturally occurring outbreaks of colibacillosis of calves are briefly reported and discussed with reference to the epidemiology of the disease. The following serotypes were recorded as being associated with these outbreaks: O9.A (type RVC118), O9.A (type RVC1798), O15.K?, O35.K?, O78.K80(B), O86.B7, O103.K(B)?, O117.K?, O137.K79(L).

Three serotypes, isolated from the udder secretions of cows with mastitis, were O9.A (type RVC1798), O24.K? and O78.K80(B).

## ACKNOWLEDGMENTS

Thanks are due to Professor R. Lovell for his advice and encouragement; to P. L. Ingram of the Royal Veterinary College, and Dr. J. H. B. Roy and his colleagues of the National Institute for Research in Dairying for supplying a wealth of material from which some of the above serotypes were first isolated; to Dr. Joan Taylor for supplying type strains of *E. coli* recovered from cases of infantile gastro-enteritis; to Dr. F. Orskov of Copenhagen who examined strain O137.K79 serologically, and to the Agricultural Research Council who provided a grant for the prosecution of this work.

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## APPENDIX

1.* *Co2/54* Cultures from the carcases of two three-day-old calves on the same farm. *E. coli* was recovered from all sites, but the strains could not be identified serologically though they were identical in their fermentation reactions. All animals were home bred, received colostrum and were pail fed. There were losses last season as well.

2. *Co3/54* Two-day-old calf. Culture from heart blood only was sent. This yielded a strain of *E. coli* which could not be identified serologically. There were lesions of septicaemia. This was the third calf to die within forty eight hours of birth and all were kept in the same box.

3. *C13/54* Fourteen-day-old calf which scoured for three days before death. An unidentifiable strain of *E. coli* was recovered from all tissues. There had been two other recent losses. One calf was bought in and died within forty eight hours.

4. *C23/54* Twenty seven-day-old calf which suckled for five days but refused to drink from the bucket at first. There was intermittent scouring with blood. A serologically unidentifiable strain of *E. coli* was isolated from the intestinal contents. Mesenteric lymph nodes and heart blood were sterile. Examination post mortem revealed an acute catarrhal enteritis and congestion of the mesenteric lymph nodes.

5. *C71/54* Twenty eight-day-old calf which died with a slight scour within three days of showing symptoms. *E. coli* was recovered from all tissues, but only one culture was submitted for examination and this did not react with any of the typing sera. The animal was pail fed since birth and was given colostrum. There were typical lesions of septicaemia.

6. *Co2/55* Two-day-old calf. Serologically unidentifiable *E. coli* was isolated from the ileal contents and spleen. This was the third to die at this age with acute scour and slight dysentery. There was no response to coliserum or streptomycin. It suckled but went off its food the next day. Examination post mortem showed peracute enteritis especially in the iliocaecal region with blood tinged contents.

* Numbers refer to farms mentioned in text.

7. *C19/55* One-day-old calf. This animal showed scours and weakness for a very short time only (no more than twelve hours before death) and a number of others died similarly. Coliserum was given. Examination post mortem revealed a septicaemia and 0137,K79 was isolated in pure culture from all organs. Cultures from another calf which died under identical circumstances were sent in six weeks later. The same serotype was present in all organs.

8. *C22/55* Eight days old. Home bred calf which was fed colostrum and whole milk from the bucket. There were extensive lesions of septicaemia and 035,K? was recovered from all tissues. Another calf on the same farm died two days later. Examination post mortem showed a few epi- and endo-cardial haemorrhages. 035,K? was recovered from all tissues.

9. *C24/55* Calf between two and three weeks old. Faecal swab yielded a strain of *E. coli* in almost pure culture which could not be identified serologically. Calves were bought in on this farm and most often began to scour within a week of their arrival. Many recovered with careful nursing.

10. *C28/55* One day old. Death was very sudden and there were very few post mortem signs save for an enlarged spleen. *E. coli* was recovered in apparently pure culture and large numbers from all organs. Only one culture was submitted for examination and this was identified as 0137,K79. Another one-day-old calf which died a day later had a similar history except that a severe enteritis was also present. *E. coli* was recovered from all organs and the culture submitted for examination was found to be 0137,K79.

11. *C45/55* Three-day-old calf which was one of several which died The culture of *E. coli* recovered from the carcase could not be identified serologically. No calves were bought in and all were fed colostrum and whole milk from the pail. Serum samples from the dam of this calf and from two other cows in the same herd were examined for agglutinins against the calf strain of *E. coli*.

| | O. | K. | titres |
|---|---|---|---|
| Carnival (dam) | — | — | |
| Edith | 80 | 160 | |
| Ursula | 40 | — | |

12. *C51/55* 'Young' calf which died very rapidly. Examination post mortem revealed a septicaemia and *E. coli* was recovered from all organs. One culture was sent for examination and this was O137,K79. Calves continued to die and a culture from one which died three weeks later under circumstances identical to the above was identified as O137,K79.

13. *C57/55* 'About a week old'. Received colostrum and pail fed milk. There were lesions of septicaemia and *E. coli* was recovered from all sites. The strain did not react with any of the typing sera.

14. *1925* 'About a week old'. Although obtained from a different farm, the details were otherwise exactly as above.

15. *C62/55* Twelve days old. Bought in at three days. Death was very sudden, but there were no lesions of septicaemia. *E. coli* was recovered from liver, spleen, kidney and heart blood, but could not be typed serologically. A few calves, mainly home-bred, had died recently.

16. *C108/55* Two days old. Home bred, received colostrum and was fed four times daily. There were lesions of septicaemia, but the strain of *E. coli* isolated from the carcase could not be identified serologically. Three calves between the ages of two and seven days had died recently though there had been no recent buying-in of stock.

17. *C110/55* Four days old. Received colostrum. Examination post mortem revealed septicaemia, polyserositis, polyarthritis and slight meningitis. *E. coli* was cultured from spleen and heart blood, but could not be identified serologically.

18. *C01/56* Eleven days old. Received colostrum and pail fed whole milk. Scouring was the main sign. An untypable strain of *E. coli* was recovered from the liver, spleen, heart blood and bone marrow. This was the second loss. No calves had been bought in.

19. *C05/56* Seven days old. Home bred. Received colostrum and pail fed whole milk. Scoured for two days and appeared to recover, but was found dead. 078,K80 was recovered in pure culture from liver, spleen and bone marrow. There had been other deaths.

20. *C12/56* Seven days old. Fed colostrum and allowed to suckle. First loss of the season. *E. coli* was isolated from liver, spleen, bone marrow and stifle joint. The single culture sent for examination was O78,K80.

21. *C20/56* Four days old. Scouring badly. O9,A? (type RVC118) an untypable strain of *E. coli* and *K. aerogenes* were isolated from the faeces. Another calf in a separate pen was examined, but there was no growth on McConkey agar from a swab taken twelve hours after birth. A rough strain was isolated on the second day of life. This animal was then put in the same pen as the scouring calf and within twenty four hours was itself scouring badly. O9,A? (type RVC118) was recovered in pure culture and large numbers from the faeces.

22. *C32/56* 'Young calf'. There were lesions of septicaemia and O137,K79 was recovered from liver, spleen and bone marrow. This animal was bought in together with its dam. There was no previous history of trouble.

23. *C33/56* Two days old. There were lesions of septicaemia and O137,K79 was recovered in pure culture and in large numbers from all sites cultured. There had been several cases of scour recently, most of which started within a week of birth. Some calves were pail fed milk; others were suckled. A few calves were bought in, but it was usually the home bred ones which were affected.

24. *C55/56* Ten days old. Examination post mortem showed a localized intestinal infection. *E. coli* was isolated from the mesenteric lymph nodes, but the culture was sparse and it could not be typed serologically. Many calves were bought in and there was a history of persistent losses despite the use of coliserum.

25. *C69/56* Three days old and the first loss for some time. Pail fed on whole milk and received colostrum. There was an intense gastro-enteritis on examination post mortem. *E. coli* was isolated from liver, spleen and bone marrow, but could not be identified serologically.

26. *C71/56* 'About seven days old'. Examination post mortem revealed venous congestion in all tissues with a few haemorrhages on the bladder mucosa and around the umbilical arteries. Liver, spleen and kidney normal. Contents of small and large intestines abnormally liquid and associated lymph nodes somewhat enlarged and oedematous. Most limb joints showed evidence of early serofibrinous synovitis. Cultures were made from heart blood, bone marrow and hock joint. Each gave a pure growth of O78,K80. Calves were bucket fed and received colostrum. Great attention was paid to hygiene on this farm, but there had been many losses during the last four years.

27. *C43/56* Four days old. Pail fed. There were lesions of septicaemia and *E. coli* was isolated from liver, spleen, heart blood and bone marrow. The culture sent for examination was identified as O117,K? Another four-day-old calf died on this farm eleven days later, and this was the third loss. No calves were bought in and the hygiene appeared to be satisfactory. Details exactly as for first calf.

[*Received for publication, December 12th,* 1957]

# STUDIES ON *ESCHERICHIA COLI* OF ANIMAL ORIGIN

## II. *Escherichia coli* from Young Lambs

By

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### INTRODUCTION

In 1921, Gaiger and Dalling sought to establish the cause of death of young lambs in Great Britain, numbers of which died annually from some form of enteritis. The results of their preliminary survey led them to believe that *Escherichia coli* was responsible for the condition which came to be known as 'lamb dysentery'. Later, it was shown that a member of the genus *Clostridium* was regularly associated with the disease (Gaiger and Dalling, 1923) and that protection against this organism gave protection against the disease (Dalling, Mason and Gordon 1928). In the United States, however, clostridia were rarely, if ever, associated with enteritis in young lambs (Heller, 1933; Marsh and Tunnicliff, 1938) and the results of a survey conducted by Marsh and Tunnicliff produced evidence that *E. coli* was one of several factors jointly responsible for this condition.

During the course of an investigation into natural outbreaks of colibacillosis in calves (Rees, 1958), a number of cultures of *E. coli* from lambs were examined. These strains were recovered from septicaemic animals or cases of diarrhoea where lamb dysentery did not appear to be the cause of death.

### METHODS

The strains were examined for their ability to ferment a variety of carbohydrate media, the production of indole, ability to make a buffered glucose medium acid to methyl red, production of acetylmethylcarbinol and ability to utilize sodium citrate as a sole source of carbon.

Serological examination was made according to the methods used for strains recovered from calves.

### RESULTS

All strains produced indole, gave a positive methyl-red reaction, failed to produce acetylmethyl-carbinol and were unable to grow in a medium containing sodium citrate as its only source of carbon. Such details as were supplied with the cultures are recorded below together with the results of serological examination. A number of cultures submitted without details have not been listed.

1. *C20/54.* A number of lambs in this flock died within 24 hours of birth. All had been injected with pulpy kidney serum. Treatment with coliserum cleared up a number of cases and had a prophylactic effect in subsequent births. The strain of *E. coli* submitted did not react with any of the antisera representing strains pathogenic for calves.

2. *C10/56.* A 'young' lamb, one of several that appeared to have died of septicaemia, was examined . Strains of *E. coli* isolated from the intestinal contents, liver, heart blood and lungs were culturally and biochemically identical, but could not be identified serologically. The farmer had brought his ewes into a shed to lamb down and the V.I.O. suggested that lambing down in this enclosed space tended to spread infection among the new-born animals.

3. *C35/56.* *E. coli* was recovered in pure culture from the liver, lung and heart blood of a lamb two or three days old. Examination post mortem revealed a thickened pericardium and haemorrhagic adhesions between lungs, wall of thorax and diaphragm. The liver was surrounded by a yellow fibrinous membrane which was adherent to the diaphragm and the dorsal surface of the sternum as well as the abdominal wall. The left kidney was greatly enlarged and displaced backwards in the abdominal cavity. Serological examination of the strains of *E. coli* showed them to be O24,K? and this was confirmed by cross-absorption tests.

4. *C56/56.* *E. coli* in pure culture was isolated from all organs but could not be identified serologically. The animal, whose age was not known, had been given lamb dysentery serum, but nevertheless developed diarrhoea and died a few days later. Examination post mortem revealed a severe enteritis. Attempts to isolate a *Clostridium* sp. were unsuccessful.

5. *C61/56.* Four lambs of unknown age which died were examined post mortem and *E. coli* was isolated from all organs. Material from two of these animals was submitted and yielded strains which were identified as O78,K80, and subsequently confirmed by cross-absorption tests. There was a clinical picture of 'white scours' and the death rate was alleged to be as high as thirty per cent!

6. *C45/57.* A lamb of unknown age scoured before death and revealed an acute enteritis at examination post mortem . Most lambs on this farm were inoculated against lamb dysentery but eight died in two days. Two calves also died from acute enteritis. All calves, piglets, etc. which died of enteritis or other causes were buried in the stack-yard which was also used as a lambing-pen. A strongly haemolytic strain of *E. coli* was recovered from the intestine of the one lamb and serological examination showed it to be RVC3033, a strain which has recently appeared in many cases of colibacillosis of calves, but which had not yet had its somatic and surface antigen determined. Complete identity was confirmed by means of cross-absorption tests. Anaerobic culture for toxin-producing *Cl. welchii* was negative. Of four examined post mortem, three yielded *E. coli* in pure culture and large numbers. Only cultures from the above-named lamb was sent for examination.

The strains from cases 3, 5 and 6, which had been identified serologically, were also identical with the respective type strains in regard to their abilities to ferment various carbohydrate media.

## DISCUSSION

In Great Britain, where lamb dysentery is responsible for most of the deaths of young lambs from enteritis, the possible role of *E. coli* as a potential pathogen has attracted little attention. Gaiger and Dalling (1921) found that they could reproduce a form of enteritis

in lambs by feeding mixed cultures of *Cl. welchii* and *E. coli* but not if these organisms were fed singly. Later, Dalling, *et al.* (1928) showed that it was only necessary to give protection against the anaerobe by means of antitoxin injections to prevent lamb dysentery in at least 99 per cent of cases. In the United States, however, clostridial enteritis of lambs is very rare indeed and yet losses from a form of infectious enteritis occur (Heller, 1933; Marsh and Tunnicliff, 1938).

More recently, Roberts (1957) has described an outbreak of disease of lambs in Australia in which the general picture was that of white scours of calves with the addition of a meningeal infection. Increased susceptibility of lambs due to the failure to receive colostrum was put forward as a very important causative factor, though the lambs in the outbreak described were said to have suckled. The organism isolated by Roberts from the liver, small intestine and C.S.F. of one of his lambs had the same somatic antigen (O78) as the strain recovered from case No. 5. This latter strain has occurred more frequently in colibacillosis of calves in the British Isles than any other serotype.

## CONCLUSIONS

Three serotypes of *Escherichia coli viz.* O78,K80; O24,K? and RVC3033 (antigens not yet determined), already known to be pathogenic for calves, are recorded from cases of disease of young lambs.

## ACKNOWLEDGMENTS

I should like to thank Professor R. Lovell for his continued advice during the course of this work and the many people who sent material for examination. The Agricultural Research Council provided a grant which made this investigation possible.

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[*Received for publication, December 12th,* 1957]

# OBSERVATIONS ON EXPERIMENTAL PARASITIC BRONCHITIS IN CALVES AND THE RESPONSE TO DIETHYLCARBAMAZINE

By

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## INTRODUCTION

Parker (1957) demonstrated the potential value of oral Diethylcarbamazine against early lungworm infestation and pointed out the need for further work to discover the optimum method of treatment. In pursuance of this an experiment was designed to test lower dose rates and compare the efficacy of oral and intramuscular routes by means of more critical observations.

## METHODS

The experimental animals were Ayrshire bull calves (17) purchased from one farmer when a few days old, reared by an early weaning method similar to that of Preston (1956), and then housed and managed as described previously (Parker, 1957).

When $3\frac{1}{2}$ to 4 months old each calf was given 10,000 infective *Dictyocaulus viviparus* larvae by stomach tube. Sixteen of the calves were then divided into four equal groups according to weight and a seventeenth, considerably small, calf (159lb) was added to the control group.

| *Group* | *No. calves* | *Larvae given* | *Dose of drug* | *Route* | *Duration of treatment* |
|---|---|---|---|---|---|
| I | 5 | 10,000 | — | — | — |
| II | 4 | 10,000 | 10mg/lb B.W. | Oral | On 14th, 15th, 16th, 17th and 18th days. |
| III | 4 | 10,000 | 10mg/lb B.W. | I/m | On 14th, 15th, 16th, 17th and 18th days. |
| IV | 4 | 10,000 | 1mg/lb B.W. | I/m | On 14th, 15th, 16th, 17th and 18th days |

Diethylcarbamazine citrate is readily soluble and was freshly dissolved (10 per cent W/V) in sterile distilled water for administration.

The general clinical picture, including respiratory rates was observed throughout and faecal larval counts performed frequently: weights were recorded weekly.

To test the development of immunity two calves from each of groups I, II and III were challenged with 15,000 infective larvae on the 44th day. The experiment was terminated on the 82nd day when all animals were slaughtered and examined post-mortem.

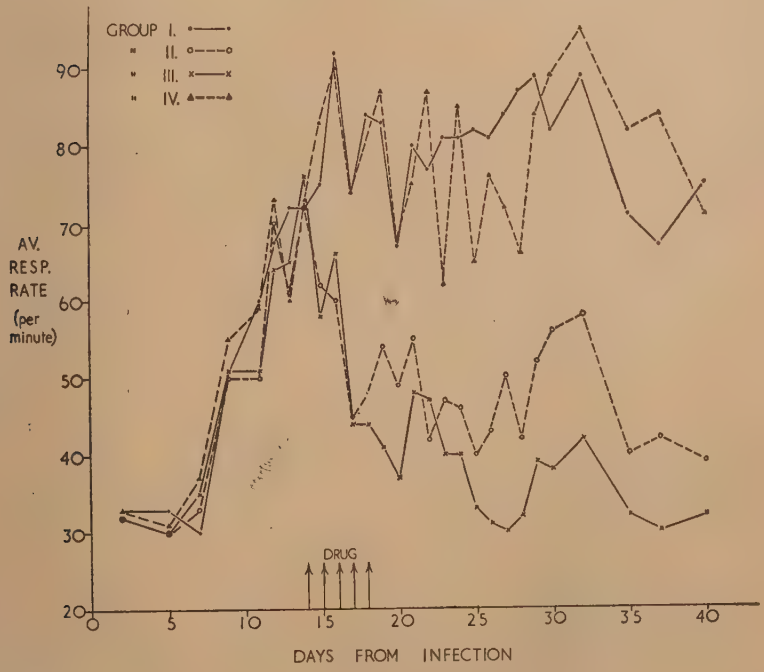
## RESULTS

Rearing was uneventful apart from the occurrence of chronic intermittent coughing in all calves commencing at 4 to 6 weeks of age and continuing in varying but slight degree throughout the period of the experiment. The severity of the disease in the smallest calf (no. 17) in the control group was so much greater than in the other calves that it is excluded from the general results and group averages.

### *Clinical Observations*

*The respiratory rate* of all calves increased from about the 7th day rising to peak figures around twice normal at about the 14th day (Fig. 1). This phase was accompanied by some depression, anorexia and a little coughing. In groups II and III the respiratory rates fell continuously from the first day of treatment to levels more or less normal by the 17th to 21st day, and this fall was paralleled by improving spirits and appetite. In groups I and IV tachypnoea continued with coughing more prevalent and little evidence of a decline until the end of the 6th week. The appetite of these calves was regained slowly.

Fig. 1

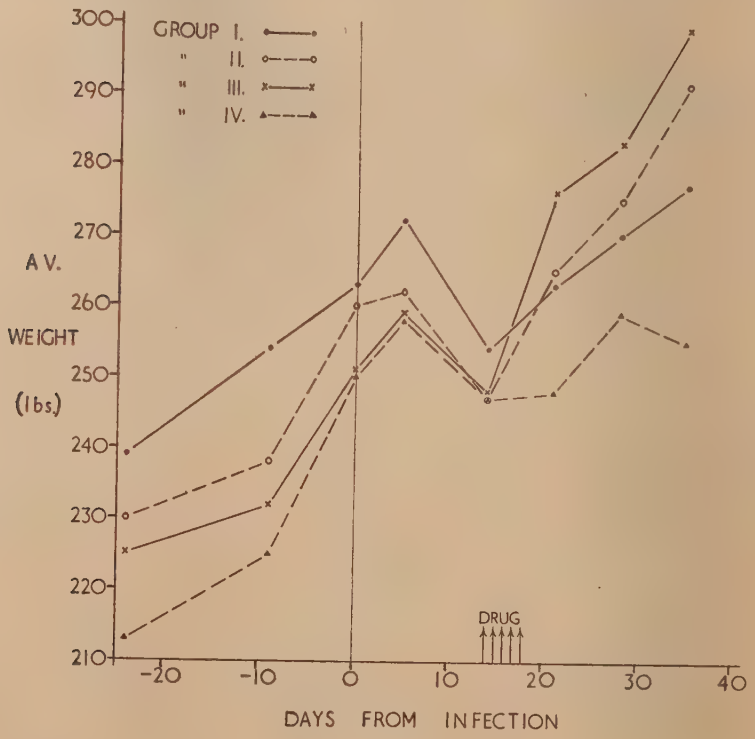



The respiratory reaction to infection and treatment.

*Body weight.* A fall of the order of 5 per cent occurred in all groups during the second week of infestation (Fig. 2). Subsequently each group gained steadily but at different rates up to the time of splitting the groups for challenge (35th day). Groups II and III grew more rapidly, the average individual daily gain over the 3rd, 4th and 5th weeks being 2·1 lb. and 2·4 lb. respectively compared with 1·1 lb. and 0·4 lb. in groups I and IV. The difference between groups declined from this time in unchallenged calves to insignificance by the end of the experiment in the 12th week. A loss of weight in the 6th and early 7th week was thought to be due to scouring associated with feeding of mouldy foodstuff.

Intramuscular injection of the drug was followed by a temporary reaction which lasted for a few minutes only and consisted of local muscular twitching around the site of injection and evidence of pain.

Fig. 2




The effect of infection and treatment on body weight.

*Faecal Examinations*

Larvae first appeared on the 21st day and were present in all calves by the 23rd day except in the case of calves of group III

TABLE 1

FAECAL LARVAL COUNTS

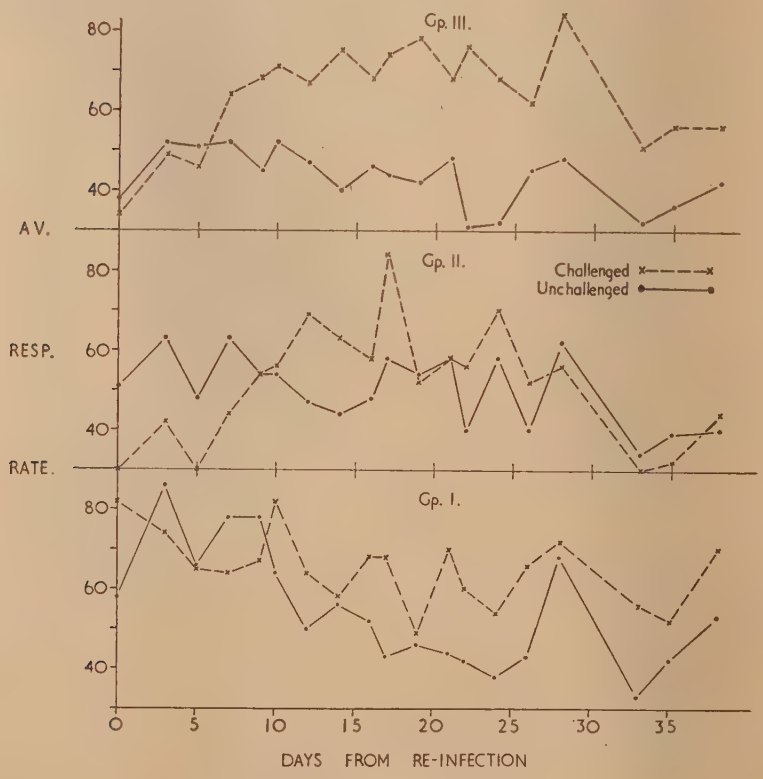
| | *Day* | *16* | *20* | *21* | *23* | *25* | *27* | *29* | *32* | *35* | *37* | *40* | *44* | *47* | *51* | *54* | *61* | *66* | *68* | *70* | *72* | *75* | *77* | *79* | *82* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group I | (4) | 0 | 0 | 0 | 6 | 50 | 400 | 100 | 50 | 2 | 50 | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | F |
| | (18) | 0 | 0 | 0 | 7 | 250 | 300 | 200 | 200 | 50 | 150 | 100 | 1 | 1 | F | 0 | 0 | 0 | 0 | 0 | 0 | F | F | F | F |
| | 12 | 0 | 0 | 0 | 32 | 100 | 600 | 150 | 250 | 9 | 50 | 3 | 1 | 1 | 0 | 0 | — | 0 | — | — | 0 | — | — | — | 0 |
| | 13 | 0 | 0 | 0 | 22 | 500 | 550 | 250 | 300 | 50 | 50 | 5 | 1 | 1 | 0 | 0 | — | 0 | — | — | 0 | — | — | — | 0 |
| | 17 | 0 | 0 | 0 | 1 | 450 | 1200 | 1550 | 5300 | 4000 | 3400 | 3000 | 3500 | 2000 | 750 | 750 | 200 | (58 days) | | Died 60th day. | | | | | |
| Group II | (5) | 0 | 0 | 0 | F | F | F | 0 | 0 | F | 0 | 0 | 0 | 0 | — | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | F | F |
| | (10) | 0 | 0 | 0 | 1 | 50 | 8 | 5 | 1 | F | 0 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | F | F | 0 |
| | 7 | 0 | 0 | 1 | 1 | 100 | 50 | 100 | 9 | 50 | 100 | — | 2 | 1 | 2 | — | — | 0 | — | — | 0 | — | — | — | 0 |
| | 6 | 0 | 0 | 1 | 5 | 100 | 8 | 2 | 6 | 1 | 2 | — | | 0 | — | 0 | — | 0 | — | — | 0 | — | — | — | 0 |
| Group III | (8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | F | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | F | F | F |
| | (9) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | F | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | F | 3 | 2 | F | F |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | F | 0 | 0 | — | 0 | 0 | 0 | 0 | — | 0 | 0 | — | 0 | — | — | — | 0 |
| | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | F | 1 | 0 | 0 | 0 | 0 | — | 0 | 0 | — | 0 | — | — | — | 0 |
| Group IV | 11 | 0 | 0 | 1 | 100 | 400 | 100 | 100 | 350 | 100 | 6 | 36 | 4 | 3 | 0 | 0 | — | 0 | — | — | 0 | — | — | — | 0 |
| | 14 | 0 | 0 | 2 | 1 | 8 | 10 | 50 | 10 | 1 | 50 | 50 | 50 | 1 | 0 | 0 | — | 0 | — | — | 0 | — | — | — | 0 |
| | 16 | 0 | 0 | 0 | 6 | 350 | 450 | 150 | 1100 | 1400 | 800 | 1550 | 400 | 150 | 150 | 3 | — | 0 | — | — | 0 | — | — | — | 0 |
| | 15 | 0 | 0 | 5 | 100 | 350 | 50 | 50 | 350 | 50 | 26 | 50 | 2 | 2 | 0 | 0 | — | 0 | — | — | 0 | — | — | — | 0 |

( ) = Calves challenged on 44th day with 15000 larvae
— = No sample
0 = No larvae
F = Fraction of a larva p.g.

*Note.*—Low larval counts obtained by 10g. Method (Parfitt, 1955)
Other larval counts obtained by 1g. McMaster.

(Table 1). Thereafter the general pattern of larval production in groups I and IV was similar, counts rising to a peak a week or so following patency and tending to decline to none or to very low figures by the 6th to 7th week. In group II the same pattern was followed but the counts were very much lower, not exceeding 100 e.p.g. Larvae were not found in faeces of calves of group III until the 32nd day and were absent after the 40th, never exceeding 1 p.g. during this period.

Fig. 3



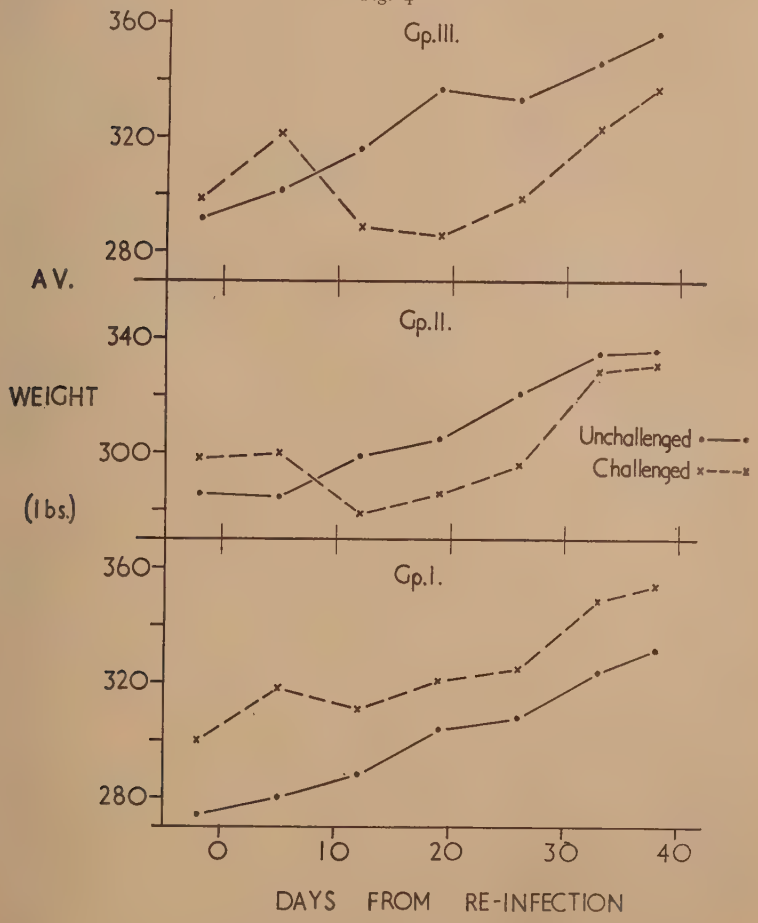

The respiratory reaction to challenge.

*Effect of Challenge*

About a week after the administration of 15,000 larvae on the 44th day tachypnoea became evident in challenged calves in groups II and III and increased progressively during the following week, remaining elevated for the next two weeks or so (Fig. 2). This was more pronounced in group III than in group II. The respiratory rates in group I were high initially and there was little

change in the general tendency to decline. The respiratory reaction was accompanied by some depression and anorexia together with a temporary fall in weight proportional to the severity of the clinical symptoms, groups I, II and III being affected in ascending order. Larvae reappeared in the faeces of all challenged calves 26 days or later after dosing but numbers did not exceed 1 p.g.

Fig. 4




The effect of challenge on body weight.

*Post-mortem Examinations*

The majority of calves killed on the 82nd day showed quite extensive localised lung lesions, though no worms were recovered from washings or ribboned lung tissue. Each lesion consisted

of one or more consolidated lobules adjacent to bronchioles containing muco-pus. Pulmonary lymph glands were enlarged, the degree being directly related to the extent of the lung lesions in the individual animal.

Sections prepared from the consolidated lung of each calf revealed similar changes in varying degrees, but without definite differences or specific change attributable to the challenge. The alveoli showed epithelialisation with diffuse eosinophilic infiltration and cellular exudation into some of the air spaces. Some of the bronchioles contained similar exudate and these also had a moderate infiltration of the epithelium. The lumina of other bronchioles were obliterated by fibrous proliferation. There was fibrosis and eosinophil-rich exudate in the septa and around the bronchioles. In all sections dense lymphocytic cuffs were present around most of the bronchioles. Sections prepared from the pulmonary lymph glands showed changes related in severity to the degree of enlargement consisting of hyperplasia, oedema and fibrosis of the trabeculae with eosinophilic infiltration, and medullary infiltration by macrophages.

The more severe clinical reaction seen in the smallest calf (no. 17) consisted of a higher respiratory rate with periodic change to abdominal type, together with a fall in body weight which was continuous from the end of the 1st week until death occurred on the 60th day. Faecal larval counts were higher and the output more prolonged (Table 1). At post-mortem examination there was a very severe emphysematous pneumonia and dilatation of the heart; 798 whole worms were recovered from the lung washings.

## DISCUSSION

From the economic point of view successful treatment of husk requires not merely that the calf shall live, but also that normal weight gains shall be resumed even though there has not been complete resolution of lung lesions. In assessing the value of any line of treatment, the severity of the attack is an important consideration.

Parasitic bronchitis may be classified from the clinical angle as:— (1) *very severe* in which deaths occur before patency, following very heavy larval infection; (2) *severe* in which deaths occur after patency, following a heavy larval invasion; (3) *moderate* covering patent infestation without deaths, following a moderate larval invasion; (4) *non-patent* covering larval invasion causing a hypersensitive reaction of the "self-cure" type (Michel, 1956) in partially immune animals.

The disease in the present experiment was of the third type and the main clinical reaction was seen in the second week following infection, an observation also made by Rubin and Lucker (1956). This reaction occurs during larval migration through the lung tissue and corresponds to the early "alveolar-bronchial phase" of Jarrett.

McIntyre, Jennings, Mulligan and Urquhart (1957b). Treatment (25mg/lb. orally for five days) applied at the end of this stage on the 14th day had aborted "very severe" infestation in an earlier experiment (Parker, 1957). A similar effect was obtained in the current trial with 10mg. either orally or intramuscularly, with evidence of slight advantage to the latter route, while 1mg. intramuscular was ineffective. Response to treatment, judging from its rapidity and the subsequent small numbers of faecal larvae, must have been the result of the elimination of the young worms. The lung lesions which had already been produced showed a persistence like that following natural elimination (Jarrett, McIntyre and Urquhart, 1957a). Their extent was insufficient to affect the development of treated calves as compared with the control animals in which the infestation continued. With more serious disease one would expect this difference between treated and untreated to be greater. Removal of worms at a later stage would probably be less beneficial because of the more advanced persisting lesions. Moreover, it seems likely that the more chronic reaction associated with mature infestations might act as a barrier to drug penetration and to the physical elimination of worms under the influence of the vermifuge. However, much higher doses of diethylcarbamazine have been used successfully against adult *D. filaria* infestation in sheep (Ozerskaya, 1953), and the application of the drug to calves is being investigated.

Qualitatively, the lesion present on the 82nd day in all calves resembled the histological picture described by Jarrett *et al.* (1957a) except for the well-marked peribronchiolar lymphoid cuffing. The common occurrence of this lesion in field husk has been recorded by the same authors (Jarrett *et al.* 1953), and it seems likely that our animals were intercurrently infected which may well have enhanced the effects of the lungworms or retarded the resolution of the parasitic lesions.

Immunity appeared to be of a lower grade in calves successfully treated than in animals which had attained natural "self-cure", in that the challenge induced a type 4 reaction in the treated calves. This would probably have little significance in calves subject to reinfection, but the observation offers direct evidence that mature lungworm infestation plays a part in eliciting immunity as well as the passage of larvae through mesenteric glands.

## CONCLUSIONS

Diethylcarbamazine in daily doses of 10mg/lb. body weight for five days commencing 14 days after infection, effectively terminated a 'moderate' lungworm infestation.

Intramuscular administration was only slightly superior to the oral route.

Doses of 1mg/lb. intramuscularly were ineffective.

The clinical response to treatment commenced immediately and

resulted in a superior weight gain over the period corresponding to the patent period.

Reaction to challenge on the 44th day suggested that immunity in treated calves was of a lower order than in animals which had experienced patent infection.

Persisting parasitic lesions were present in both treated and untreated calves at post-mortem examination on the 82nd day.

ACKNOWLEDGMENTS

We are indebted to Mr. J. F. Michel of Weybridge for supply of larvae for the experiment and to Messrs. Burroughs Wellcome for the supplies of "Banocide" (Diethylcarbamazine). We are also grateful to Dr. E. L. Taylor of Weybridge and Mr. S. L. Hignett of the Wellcome Veterinary Research Station, Frant, for their interest and advice, and to the staff of the Veterinary Investigation Centre at Wolverhampton for their willing assistance.

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[*Received for publication, December 23rd,* 1957]

# A COMPARATIVE STUDY OF THE INCIDENCE OF VACUOLATED NEURONES IN THE MEDULLA FROM APPARENTLY HEALTHY SHEEP OF VARIOUS BREEDS

By

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## INTRODUCTION

In a previous contribution Zlotnik and Rennie (1957) showed that vacuolated neurones were present in the medullas of 61 to 89 per cent of apparently healthy sheep mainly of the Cheviot breed. In a further paper one of the authors (Zlotnik, 1957) compared the number of vacuoles present in 54 serial sections from the medulla of scrapie animals with those of apparently healthy sheep and found that although vacuoles could be demonstrated in 70 per cent of healthy sheep, their number was much less than in sheep with scrapie. Since most of these animals were Cheviots, a breed known to be affected with scrapie, it was decided to ascertain the incidence of vacuolation in other breeds from this country and from abroad. Through the courtesy of the U.S. Department of Agriculture, 29 brains of sheep from flocks known to be free from scrapie were secured for examination. The British breeds investigated were the Blackface and Welsh Mountain, chosen since such sheep are not known to be naturally affected with scrapie.

## MATERIAL AND METHODS

The material consisted of medullas from 60 apparently healthy sheep of the following breeds: Twenty-one Blackface hoggs about 1 year old; ten Welsh Mountain hoggs about 1 year old; ten Crossbred hoggs about 1 year old (Southdown, Hampshire, Shropshire and Merino) from U.S.A.; ten Columbia adult sheep (U.S.A.); eight Rambouillet adult sheep (U.S.A.); one Hampshire adult sheep (U.S.A.).

The brains were removed after destruction of the animals. The medullas were fixed in 10 per cent formol-saline and post-fixed in mercuric chloride formol. Three blocks were cut from each medulla as follows: "A"=Cross section of anterior medulla—trapezoid body. "B"=Cross section through both olives immediately posterior to the trapezoid body —about $\frac{1}{4}''$ thick. "C"=Cross section through the medulla $\frac{1}{4}''$ thick immediately posterior to block "B".

Serial sections $7\mu$ in thickness were cut from each block and stained with haematoxylin and eosin. Eighteen consecutive sections of each block were examined microscopically, a total of 54 sections from each medulla, and all definite vacuoles were counted.

## RESULTS

The results of microscopical examination of the medulla from the various groups of animals are summarised in Tables 1 to 4.

*Blackface sheep.* Vacuoles were found in all 21 medullas, the lowest count in 54 sections being 3 vacuoles (average per section 0·055). The highest count was 76 vacuoles (1·4 vacuoles per section) in one animal, and the next highest was 32 in 54 sections (0·59 per section). The majority of the vacuoles were present in block "C", while block "A" contained the smallest number. Thus in 10 animals no vacuoles were present in block "A", only 5 medullas had no vacuoles in cut "B" and one had none in cut "C" (Table 1).

TABLE 1

VACUOLATION OF NEURONES

Blackface Sheep (about 1 year old).

| *Serial no.* | *Total no. of vacuoles in 18 consecutive sections* | | | *Total no. of vacuoles in 54 sections* | *Average no. of vacuoles per section* |
|---|---|---|---|---|---|
| | *Cut "A"* | *Cut "B"* | *Cut "C"* | | |
| C. 127 | 2 | — | 2 | 4 | 0·074 |
| C. 128 | — | 3 | 1 | 4 | 0·074 |
| C. 129 | 1 | 8 | 7 | 16 | 0·3 |
| C. 130 | — | 1 | 7 | 8 | 0·148 |
| C. 131 | 4 | 4 | 1 | 9 | 0·166 |
| C. 132 | — | 4 | 16 | 20 | 0·37 |
| C. 133 | — | — | 32 | 32 | 0·59 |
| C. 134 | — | 16 | 5 | 21 | 0·39 |
| C. 135 | 3 | 2 | 22 | 27 | 0·5 |
| C. 136 | 4 | 9 | 7 | 20 | 0·37 |
| C. 137 | 4 | 5 | 67 | 76 | 1·4 |
| C. 138 | 1 | 7 | — | 8 | 0·148 |
| C. 139 | 3 | 3 | 4 | 10 | 0·185 |
| C. 140 | — | — | 4 | 4 | 0·074 |
| C. 141 | — | — | 3 | 3 | 0·055 |
| C. 142 | 3 | — | 25 | 28 | 0·52 |
| C. 143 | 2 | 2 | 3 | 7 | 0·13 |
| C. 144 | — | 15 | 9 | 24 | 0·44 |
| C. 145 | — | 7 | 3 | 10 | 0·185 |
| C. 146 | — | 2 | 17 | 19 | 0·35 |
| C. 147 | 3 | 9 | 1 | 13 | 0·24 |

*Welsh Mountain sheep.* Vacuoles were found in only 6 medullas. The lowest count was 1 vacuole in 54 sections (0·018 per section), the highest was 39 vacuoles (0·72 per section). Cut "A" contained the smallest number of vacuoles, only single vacuoles being found in 3 cases, and cut "B" had the highest number (Table 2).

*Crossbred hoggs* (U.S.A.). Vacuoles were found in 90 per cent of medullas, the smallest number being in cut "A" and the highest in cut "B". The lowest count was 1 vacuole in 54 sections (0·018 per section) the highest 27 (0·5 per section). Vacuoles were present in 2 only of cut "A" and 4 of cut "C" (Table 3).

*Columbia* (U.S.A.). Seven of 10 animals showed vacuoles, the lowest count being 2 vacuoles in 54 sections (0·037 per section), the

highest 21 vacuoles (0·39 per section). Most vacuoles were present in block "C"; block "A" contained the smallest number (Table 4).

TABLE 2

VACUOLATION OF NEURONES

Welsh Mountain Sheep.

| *Serial no.* | *Total no. of vacuoles in 18 consecutive sections* | | | *Total no. of vacuoles in 54 sections* | *Average no. of vacuoles per section* |
|---|---|---|---|---|---|
| | *Cut "A"* | *Cut "B"* | *Cut "C"* | | |
| C. 217 | — | — | — | Nil | Nil |
| C. 219 | — | — | — | Nil | Nil |
| C. 220 | — | 23 | 16 | 39 | 0·72 |
| C. 237 | — | 10 | — | 10 | 0·185 |
| C. 239 | 1 | 2 | 3 | 6 | 0·11 |
| C. 240 | — | — | — | Nil | Nil |
| C. 241 | — | 9 | — | 9 | 0·166 |
| C. 242 | — | — | — | Nil | Nil |
| C. 243 | 1 | 15 | 5 | 21 | 0·39 |
| C. 244 | 1 | — | — | 1 | 0·018 |

TABLE 3

VACUOLATION OF NEURONES

Crossbred Hoggs—Southdown, Shropshire, Hampshire and Merino (1 year old).

| *Serial no.* | *Total no. of vacuoles in 18 consecutive sections* | | | *Total no. of vacuoles in 54 sections* | *Average no. of vacuoles per section* |
|---|---|---|---|---|---|
| | *Cut "A"* | *Cut "B"* | *Cut "C"* | | |
| C. 277 | — | 8 | — | 8 | 0·148 |
| C. 278 | — | — | — | Nil | Nil |
| C. 279 | — | 20 | — | 20 | 0·37 |
| C. 280 | — | 14 | — | 14 | 0·26 |
| C. 281 | — | 22 | 5 | 27 | 0·5 |
| C. 282 | 2 | — | 2 | 4 | 0·074 |
| C. 283 | — | 1 | — | 1 | 0·018 |
| C. 284 | — | 2 | 2 | 4 | 0·074 |
| C. 285 | 3 | 9 | 3 | 15 | 0·28 |
| C. 286 | — | 4 | — | 4 | 0·074 |

*Rambouillet* (U.S.A.). Vacuoles were present in 7 of 8 medullas, the highest count being 18 vacuoles in 54 sections (0·33 per section) and the lowest 1 vacuole (0·018 per section). Block "A" contained the lowest number and block "C" the highest (Table 4).

*Hampshire* (U.S.A.). Only 1 animal was available for examination and 6 vacuoles were found in 54 sections (0·11 per section). The vacuoles were equally divided between blocks "B" and "C", none being present in block "A" (Table 4).

TABLE 4

VACUOLATION OF NEURONES

10 Columbia, 8 Rambouillet and 1 Hampshire.

| *Serial no.* | *Total no. of vacuoles in 18 consecutive sections* | | | *Total no. of vacuoles in 54 sections* | *Average no. of vacuoles per section* |
|---|---|---|---|---|---|
| | *Cut "A"* | *Cut "B"* | *Cut "C"* | | |
| | | | — Columbia — | | |
| C. 320 | — | — | — | Nil | Nil |
| C. 321 | — | — | — | Nil | Nil |
| C. 322 | — | — | 2 | 2 | 0·037 |
| C. 324 | — | 5 | 4 | 9 | 0·166 |
| C. 327 | — | — | 11 | 11 | 0·2 |
| C. 329 | — | 1 | 7 | 8 | 0·148 |
| C. 333 | — | — | — | Nil | Nil |
| C. 334 | 5 | 14 | 2 | 21 | 0·39 |
| C. 335 | 2 | — | 2 | 4 | 0·074 |
| C. 337 | 3 | — | 4 | 7 | 0·13 |
| | | | — Rambouillet — | | |
| C. 319 | — | — | — | Nil | Nil |
| C. 323 | 1 | 3 | 9 | 13 | 0·24 |
| C. 325 | — | 18 | — | 18 | 0·33 |
| C. 326 | 2 | 5 | 3 | 10 | 0·185 |
| C. 328 | — | 1 | 3 | 4 | 0·074 |
| C. 330 | — | — | 3 | 3 | 0·055 |
| C. 332 | — | 1 | — | 1 | 0·018 |
| C. 336 | 1 | — | 9 | 10 | 0·185 |
| | | | — Hampshire — | | |
| C. 331 | — | 3 | 3 | 6 | 0·11 |

## DISCUSSION

The results of this survey show that vacuolation in the medulla of apparently healthy sheep is by no means confined to the Cheviot breed. Vacuoles can be found not only in breeds susceptible to scrapie, but also in breeds where scrapie is unknown or in sheep from flocks where scrapie has never been recorded. The proportion of medullas showing vacuoles in the various breeds seems very similar to that reported for Cheviots, except for the Blackface sheep where vacuoles were found in all the medullas examined. (Welsh Mountain 60 per cent; American Crossbred hoggs 90 per cent; Columbia 70 per cent and Rambouillet 87 per cent). The part of the medulla least affected by vacuolation is the trapezoid body (cut "A"), but the incidence of vacuoles in the other parts varies according to breed. In the Cheviot (Zlotnik, 1958) cut "C" is the most severely affected and the same is true for Blackface and

Columbia sheep. In the Rambouillet the numbers are more or less equal in cuts "B" and "C". Finally, in the Welsh Mountain and in the American Crossbred hoggs the highest number is found in cut "B".

Although vacuoles have been recorded in the medullas of apparently healthy sheep (Holman and Pattison, 1943; Bosanquet, Daniel and Parry, 1956; Zlotnik and Rennie, 1957) the actual numbers were first given by one of the writers (Zlotnik, 1957) for 10 sheep mainly of the Cheviot breed. According to this work, vacuoles occurred in 70 per cent of sheep, the numbers in individual sheep varying from 0·05 to 1·05 per section. The results reported in the present paper show a somewhat similar range. In the Blackface breed the numbers are somewhat higher (0·074 to 1·4 per section), while in other breeds the numbers are lower (Welsh Mountain 0·018 to 0·72 vacuoles per section; American Crossbred hoggs 0·018 to 0·5; Columbia 0·037 to 0·39; Rambouillet 0·018 to 0·33).

The significance of the vacuole counts in the medullas of apparently healthy sheep is of great importance for the diagnosis of scrapie. Of the 70 apparently healthy sheep examined (including those of Zlotnik, 1957), the average number of vacuoles exceeds an average of 1 per section in only two medullas, in another 6 sheep the number exceeds 0·5, while in the remainder the range is from below half a vacuole per section to nil.

## CONCLUSIONS

Vacuolated neurones in the medulla of apparently healthy sheep can be found even in breeds unaffected by natural scrapie.

The number of vacuoles throughout the medulla is generally very low and only in exceptional cases exceeds one vacuole per section.

The significance of these results in the laboratory diagnosis of scrapie is discussed.

## ACKNOWLEDGMENTS

The authors wish to thank Dr. J. T. Stamp, Director of the Moredun Institute for his interest in the work and for reading the manuscript. Special thanks are due to Dr. H. W. Johnson, Director of Animal Disease and Parasite Division, U.S.A., and Dr. B. T. Simms, Assistant Administrator, Production Research, U.S.A., for the brains from American breeds of sheep; and to Messrs. C. M. Edwards, B.V.Sc., M.R.C.V.S., of Bangor, and G. F. Kershaw, M.R.C.V.S., of Aberystwyth for providing brains from Welsh Mountain sheep.

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[*Received for publication, December 27th,* 1957]

# IMMUNITY AGAINST BRUCELLA INFECTION IN GUINEA PIGS PRODUCED BY THREE DIFFERENT VACCINES

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## INTRODUCTION

In a previous experiment (Jones and Alton, 1956) a killed adjuvant vaccine prepared from a rough culture of *Br. melitensis* produced the same degree of immunity in guinea pigs as a similar vaccine prepared from a smooth strain. Herzberg and Elberg (1955) reported excellent protection against *Br. melitensis* infection in guinea pigs by the use of a living avirulent strain of *Br. melitensis* which was a non-dependent mutant isolated from a streptomycin-dependent population. These results have since been confirmed in goats (Elberg and Faunce, 1957).

Unpublished data from this laboratory had shown that Strain 19 may not protect guinea pigs against challenge with recently isolated field strains of *Br. abortus* and *Br. melitensis* to the same extent as against challenge with laboratory strains even though all strains had approximately the same $ID_{50}$ in non-vaccinated guinea pigs. In view of this it seemed dangerous to compare the results of different experiments unless the same challenge strain had been used in each.

The present experiment was carried out simultaneously with one in goats reported elsewhere (Jones, Thomson and Alton, 1958), designed to compare the immunity produced by the smooth and rough adjuvant vaccines against the *Br. melitensis* challenge strain used previously in this series. It was intended to compare in guinea pigs the immunity produced by the rough and smooth adjuvant vaccines against challenge with additional strains, both *Br. abortus* and *Br. melitensis*. Two months after this part of the experiment had begun, Elberg's living vaccine was made available and we were then able to compare the efficacy of this vaccine with the adjuvant vaccines both in goats and guinea pigs. In the goat experiment, challenge infection took place four months after the living vaccine group had been inoculated and six months after the adjuvant vaccine groups had been inoculated. Some guinea pigs inoculated with the living vaccine were challenged at four months and some at six months to determine whether the immunity diminished significantly during this period. It was hoped that valuable data would be acquired on the correlation between results in goats and in guinea pigs. The opportunity was also taken of investigating the effect of storage on the rough adjuvant vaccine.

## MATERIALS AND METHODS

*Vaccination of guinea pigs.* The rough and smooth adjuvant vaccines

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described in the concurrent goat experiment (Jones, *et al.*, 1958) were inoculated into guinea pigs one day following the goat inoculations. Each guinea pig received 0·2 ml of one of the vaccines (i.e., a dose of $10^{10}$ killed organisms) subcutaneously in the right flank. A small group of guinea pigs was inoculated in the same manner with a batch of rough adjuvant vaccine (R') prepared four weeks previously and stored at 4°C. Eight weeks later the living *Br. melitensis* vaccine received from Elberg was inoculated subcutaneously into additional guinea pigs. Each animal was given 0·2 ml of a dilution calculated to be one-tenth of the goat dose. Viable counts performed, on the day of inoculation, indicated that each guinea pig received $2 \times 10^8$ organisms. Guinea pigs inoculated with living vaccine were housed until the time of challenge in a different building from those given killed vaccines.

*Challenge infection and determination of* $ID_{50}$. The four strains used for challenge infection were as follows:—

H38—the challenge strain of *Br. melitensis* used in previous goat and guinea pig experiments in Tunis (Renoux, Alton and Amarasinghe, 1957) and at Weybridge.

544—the challenge strain of *Br. abortus* used in cattle and guinea pig experiments in Britain.

6015—the challenge strain of *Br. melitensis* used in guinea pig and goat experiments in California (Herzberg and Elberg, 1955; Elberg and Faunce, 1957).

177—a field strain of *Br. melitensis* isolated two weeks previously by Alton from a goat in Malta.

The four strains, having been preserved as dried cultures, were opened and grown on serum dextrose agar. A forty-eight hour growth from the second subculture was standardized in an electric nephelometer and diluted appropriately in peptone water. Viable counts were performed on Albimi agar on the day of inoculation. One ml of the appropriate dilution was injected intramuscularly in the right hind leg of each guinea pig. Guinea pigs were challenged approximately six months after vaccination except where otherwise stated. The actual day of challenge infection with the several strains was arranged at intervals so that autopsies, which commenced six weeks after challenge, could be completed for each challenge strain within a few days.

Precrural, axillary, cervical, submaxillary and iliac lymph nodes and spleen were minced and streaked on Albimi agar containing antibiotics in the concentrations previously used (Jones and Alton, 1956). One colony from each infected guinea pig was typed. The recovery of a single colony of brucella from a guinea pig was considered to be evidence of infection of the animal. The calculated percentage of guinea pigs infected with each challenge dose, as derived from cumulative totals, was plotted on log probability paper and the $ID_{50}$ and confidence limits were determined according to the method of Bonet-Maurey, Jude and Servant (1954). The $chi^2$ test is as described by Litchfield and Wilcoxon (1949) except that the "percent expected" is obtained from the cumulative totals.

## RESULTS

The results of the experiment in which guinea pigs were challenged with strain H38 four months after inoculation of living vaccine and six months after inoculation of the adjuvant vaccines

are shown in Table 1 and Graph 1. It can be seen that the living vaccine produced significantly greater immunity than the killed vaccines as shown by the confidence limits of the living vaccine, which do not overlap those of any other. There was no significant difference in the protective power of the rough and smooth adjuvant vaccines. Storing the rough vaccine for four weeks before use (line R' in Graph 1) did not affect its immunizing capacity.

TABLE 1

$ID_{50}$ OF *BR. MELITENSIS* H38 IN VACCINATED GUINEA PIGS

Four Months After Inoculation of Living Vaccine and Six Months After Inoculation of Killed Vaccines

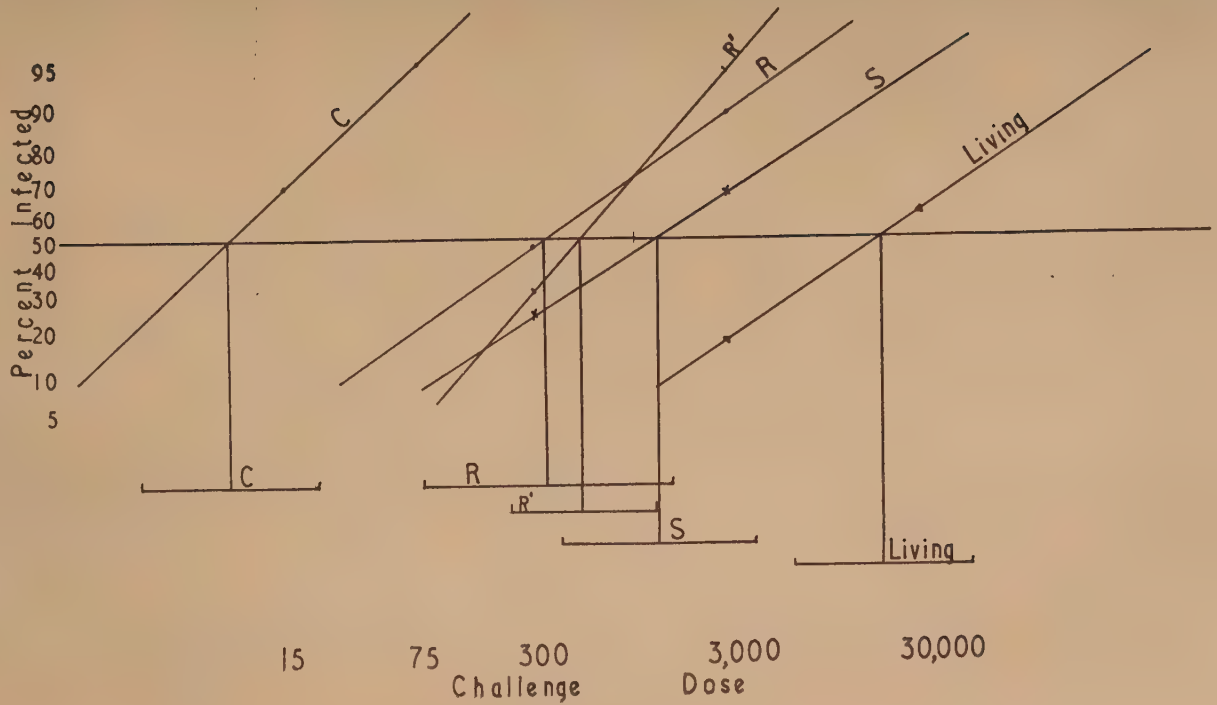
| *Vaccine* | *Challenge dose* | *Number infected* / *inoculated* | *Infection* — *Per cent observed* | *Per cent expected* | $ID_{50}$ | *Confidence limits* | *Chi² of the line** |
|---|---|---|---|---|---|---|---|
| Controls | 15 | 9/12 | 75 % | 69·2% | 7·6 | 2·6 | 0·60 |
| | 75 | 10/11 | 90·9 | 95 | | to | |
| | 300 | 12/12 | 100 | 100 | | 22 | |
| Rough | 300 | 6/11 | 54·5 | 46·1 | 340 | 75 | 0·72 |
| | 3,000 | 9/11 | 81·8 | 88·2 | | to | |
| | 30,000 | 12/12 | 100 | 100 | | 1,530 | |
| Rough (R') | 300 | 4/12 | 33·3 | 30·7 | 520 | 225 | 1·01 |
| | 3,000 | 12/13 | 92·3 | 94·1 | | to | |
| | 30,000 | 13/13 | 100 | 100 | | 1,248 | |
| Smooth | 300 | 4/12 | 33·3 | 23·5 | 1,300 | 406 | 1·57 |
| | 3,000 | 6/11 | 54·5 | 66·6 | | to | |
| | 30,000 | 12/12 | 100 | 100 | | 4,160 | |
| Living | 300 | 0/12 | 0 | 0 | 19,000 | 6,552 | 1·08 |
| | 3,000 | 3/12 | 25 | 16·6 | | to | |
| | 30,000 | 6/12 | 50 | 60 | | 55,100 | |

* In each of the groups the number of degrees of freedom is one and to indicate a good fit the chi² of the line must be less than 3·8.

The results of the experiment designed to assess the duration of immunity of the living and killed vaccines are recorded in Table 5 and Graph 5. The guinea pigs were challenged with strain H38 six months after inoculation of living vaccine and eight months after inoculation of rough vaccine. The results can be compared with those in Table 1, which demonstrates the immunity produced by these vaccines two months earlier. It can be seen that the protection afforded by the rough vaccine was approximately the same six and eight months after vaccination, whereas that of the living vaccine had decreased somewhat from four to six months. The living vaccine still gave significantly greater protection than the rough vaccine but the difference between them was not as great at this time interval as it had been two months earlier.

Graph 1

Determination of the $ID_{50}$ of Br. melitensis H38



Tables 2, 3 and 4 record the results of infections with three other brucella strains in guinea pigs which had been inoculated six months previously with rough or smooth adjuvant vaccines. There was never a significant difference in the protective power of the two vaccines. In Table 2 the numbers of infected guinea pigs in the smooth vaccine group do not fit the expected dose response line (chi² of line is greater than 3·8).

TABLE 2

$ID_{50}$ OF *BR. ABORTUS* 544

Guinea Pigs Six Months After Inoculation of Killed Vaccines

| *Vaccine* | *Challenge dose* | *Number infected* / *inoculated* | *Infection* — *Per cent observed* | *Per cent expected* | $ID_{50}$ | *Confidence limits* | *Chi² of the line** |
|---|---|---|---|---|---|---|---|
| Controls | 13 | 4/12 | 33·3% | 28·6% | 22 | 11 | 0·24 |
| | 65 | 9/11 | 81·8 | 86·6 | | to | |
| | 260 | 12/12 | 100 | 100 | | 46 | |
| Rough | 260 | 3/11 | 27·2 | 17·6 | 1,700 | 425 | 1·37 |
| | 2,600 | 6/9 | 66·6 | 60 | | to | |
| | 26,000 | 9/12 | 75 | 85·7 | | 6,800 | |
| Smooth | 260 | 2/12 | 16·6 | 9·5 | 3,600 | 973 | 5·64 |
| | 2,600 | 8/11 | 72·7 | 52·6 | | to | |
| | 26,000 | 6/12 | 50 | 72·7 | | 13,320 | |

* In each of the groups the number of degrees of freedom is one and to indicate a good fit the chi² of the line must be less than 3·8.

TABLE 3

$ID_{50}$ OF *BR. MELITENSIS* 6015

Guinea Pigs Six Months After Inoculation of Killed Vaccines

| *Vaccine* | *Challenge dose* | *Number infected* / *inoculated* | *Infection* — *Per cent observed* | *Per cent expected* | $ID_{50}$ | *Confidence limits* | *Chi² of the line** |
|---|---|---|---|---|---|---|---|
| Controls | 12 | 5/9 | 55·5% | 41·7% | 16 | 6·6 | 1·1 |
| | 62 | 8/11 | 72·7 | 81·3 | | to | |
| | 250 | 11/11 | 100 | 100 | | 38·4 | |
| Rough | 250 | 6/10 | 60 | 37·5 | 660 | 176 | 1·34 |
| | 2,500 | 6/11 | 54·5 | 66·6 | | to | |
| | 25,000 | 10/11 | 90·9 | 95·6 | | 2,475 | |
| Smooth | 250 | 5/12 | 41·6 | 35·7 | 420 | 136 | 0·42 |
| | 2,500 | 10/12 | 83·3 | 88·2 | | to | |
| | 25,000 | 12/12 | 100 | 100 | | 1,302 | |

* In each of the groups the number of degrees of freedom is one and to indicate a good fit the chi² of the line must be less than 3·8.

TABLE 4

$ID_{50}$ OF *BR. MELITENSIS* 177

Guinea Pigs Six Months After Inoculation of Killed Vaccines

| *Vaccine* | *Challenge dose* | *Number infected* / *inoculated* | *Infection* | | $ID_{50}$ | *Confidence limits* | *Chi² of the line** |
|---|---|---|---|---|---|---|---|
| | | | *Per cent observed* | *Per cent expected* | | | |
| Controls | 19 | 3/12 | 25 % | 16·6% | 70 | 32 | 1·18 |
| | 95 | 6/10 | 60 | 60 | | to | |
| | 380 | 10/12 | 83·3 | 90·5 | | 154 | |
| Rough | 380 | 3/11 | 27·3 | 17·6 | 2,000 | 714 | 1·31 |
| | 3,800 | 7/12 | 58·3 | 62·5 | | to | |
| | 38,000 | 9/10 | 90 | 95 | | 5,600 | |
| Smooth | 380 | 3/13 | 23·1 | 15· | 2,500 | 555 | 1·27 |
| | 3,800 | 7/12 | 58·3 | 58·8 | | to | |
| | 38,000 | 12/14 | 85·7 | 91·7 | | 11,250 | |

* In each of the groups the number of degrees of freedom is one and to indicate a good fit the chi² of the line must be less than 3·8.

TABLE 5

$ID_{50}$ OF *BR. MELITENSIS* H38 IN GUINEA PIGS

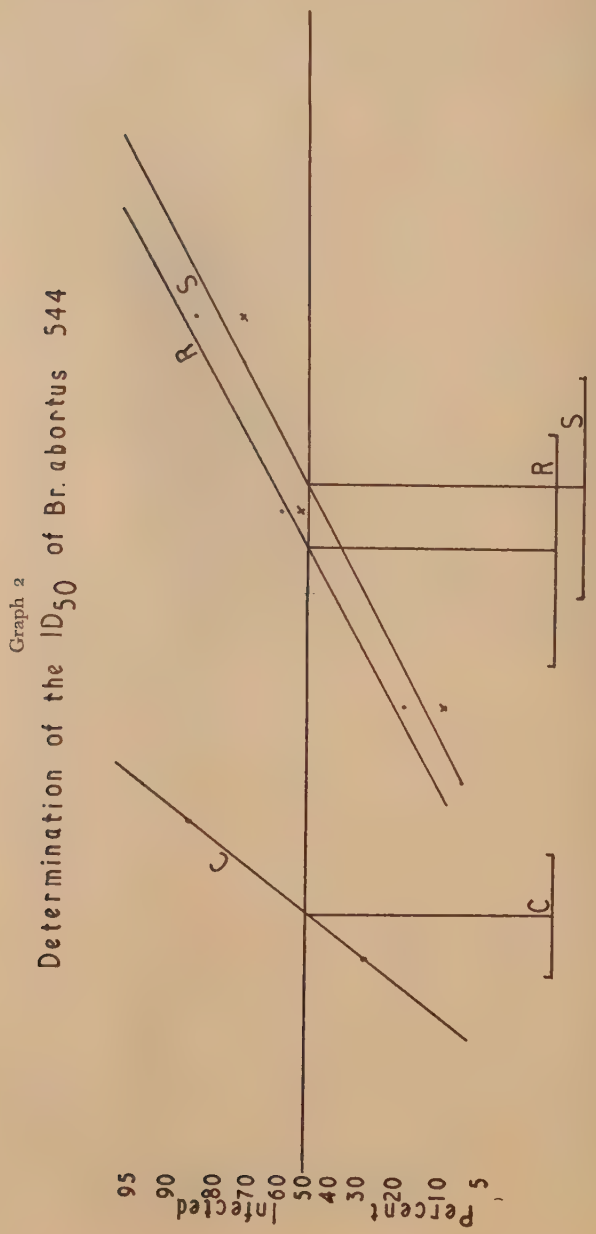
Six Months After Inoculation of Living Vaccine and Eight Months After Inoculation of Rough Vaccine

| *Vaccine* | *Challenge dose* | *Number infected* / *inoculated* | *Infection* | | $ID_{50}$ | *Confidence limits* | *Chi² of the line** |
|---|---|---|---|---|---|---|---|
| | | | *Per cent observed* | *Per cent expected* | | | |
| Controls | 5 | 2/10 | 20 % | 15·4% | 12 | 5 | 0·89 |
| | 20 | 8/10 | 80 | 76·9 | | to | |
| | 80 | 7/8 | 87·5 | 94·4 | | 30 | |
| Rough | 200 | 3/11 | 27·2 | 16·7 | 550 | 289 | 1·45 |
| | 600 | 5/10 | 50 | 53·3 | | to | |
| | 2,000 | 8/10 | 80 | 88·9 | | 1,045 | |
| Living | 2,000 | 5/11 | 45·4 | 33·3 | 4,000 | 1,538 | 1·68 |
| | 10,000 | 6/9 | 66·7 | 73·3 | | to | |
| | 60,000 | 8/9 | 88·8 | 95 | | 10,400 | |

* In each of the groups the number of degrees of freedom is one and to indicate a good fit the chi² of the line must be less than 3·8.

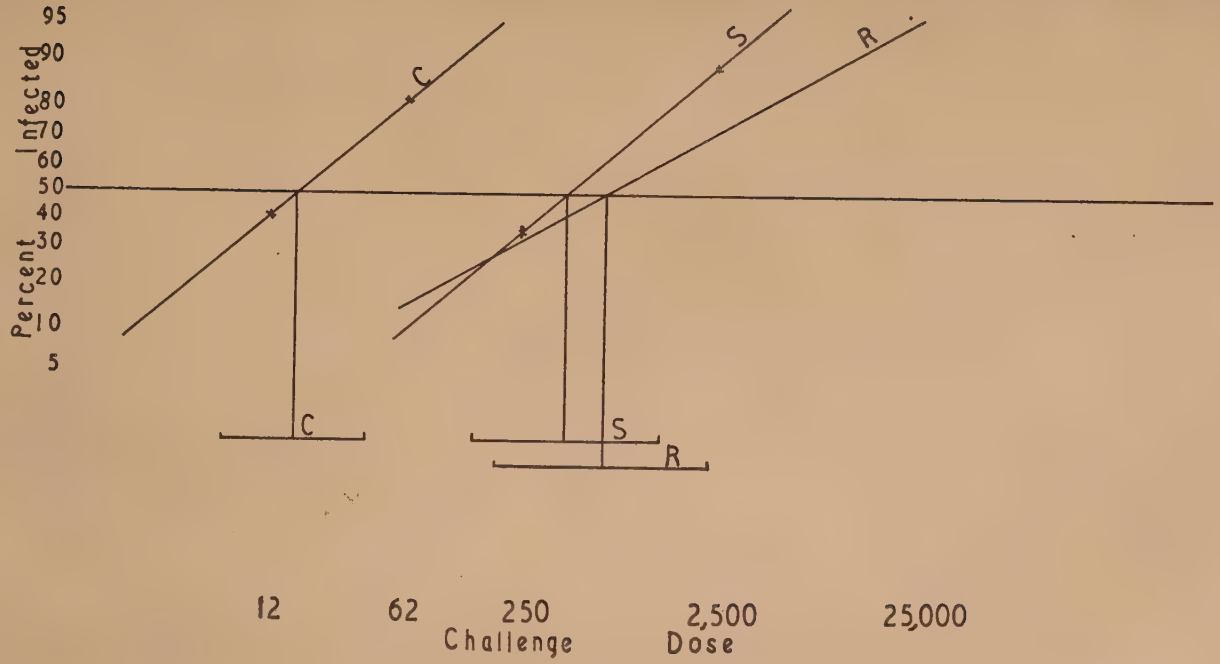
The results may be expressed in terms of the increase in $ID_{50}$ of the vaccinated over the control group for each challenge strain, provided one remembers that confidence limits are involved (Table 6). Strains 6015 and 177 were more effective in breaking down the immunity of the rough and smooth vaccines than were the other two strains. In non-vaccinated guinea pigs strains H38, 544 and

6015 had approximately the same $ID_{50}$, whereas that of 177 was significantly higher.



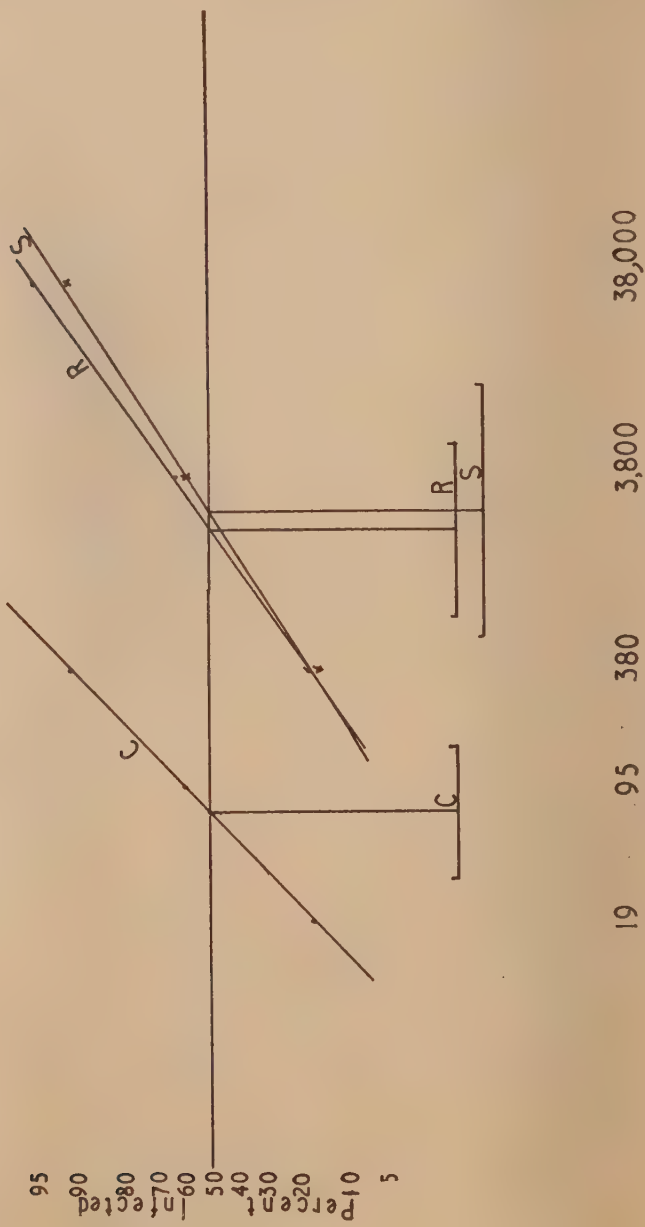
Graph 3

Determination of the $ID_{50}$ of Br. melitensis 6015



Graph 4

Determination of the $ID_{50}$ of Br. melitensis 177



Graph 5

Determination of the $ID_{50}$ of Br. melitensis H38:

6 Months after Inoculation of Living Vaccine
8 " " " " Rough "

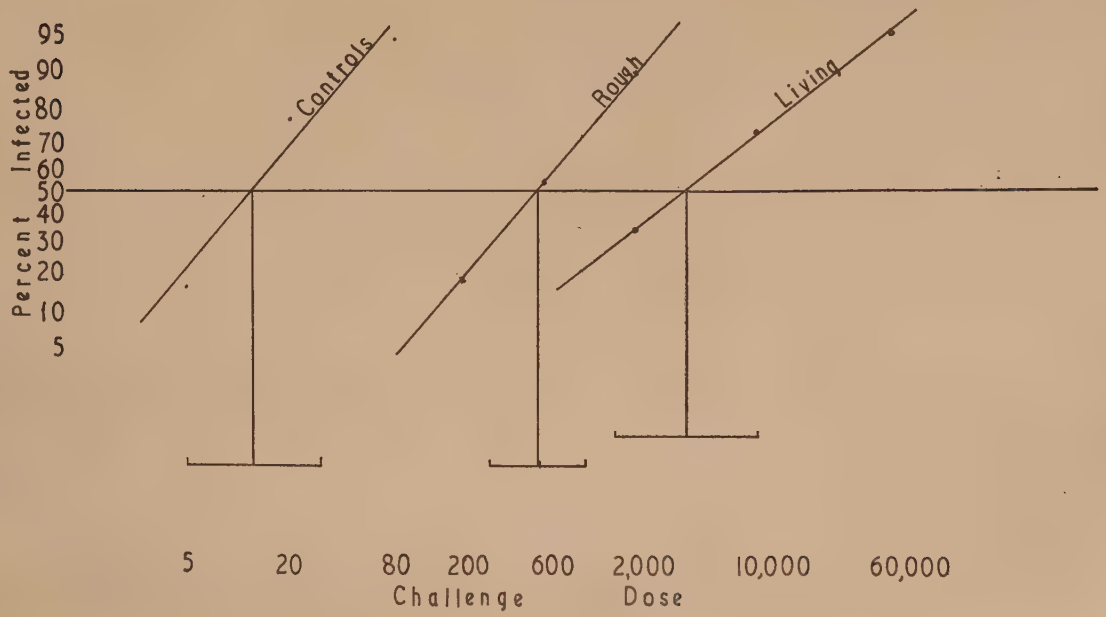


TABLE 6

| *From Table* | *Challenge strain* | *$ID_{50}$ in control groups* | *Increase in $ID_{50}$ of vaccinated over control groups for vaccine* | | |
|---|---|---|---|---|---|
| | | | *Rough* | *Smooth* | *Living* |
| 1 | H38 | 7·6 organisms | 45× | 171× | 2500× |
| 2 | 544 | 22 ,, | 77× | 164× | |
| 3 | 6015 | 16 ,, | 41× | 26× | |
| 4 | 177 | 70 ,, | 29× | 36× | |
| 5 | H38 | 12 ,, | 46× | | 333× |

## DISCUSSION

The immunity produced by a killed rough strain of *Br. melitensis* in adjuvant appears to be the same two months, six months and eight months after vaccination. In the previous study, (Jones and Alton, 1956), the $ID_{50}$ of vaccinated guinea pigs was 40 times that of controls when challenged with H38 two months after vaccination. In the present experiments it was 45 times that of controls six months after vaccination and 46 times that of controls eight months after vaccination. In contrast, the immunity produced by the living vaccine was 2,500 times that of controls when challenged with H38 four months after vaccination, and 333 times that of controls when challenged six months after vaccination. It would be interesting to know whether the immunity produced by the rough adjuvant vaccine would persist for a year or longer, and whether that produced by the living vaccine would continue to fall.

The results obtained in Table 1 compare favourably with those of the parallel goat experiment in demonstrating the superiority of the living vaccine. Guinea pigs appear to be satisfactory for the comparison of vaccines for larger animals when an $ID_{50}$ method is followed.

The challenge strain of *Br. melitensis* (6015) used by Elberg and the strain recently isolated from a Maltese goat (177) provided the most severe tests for the adjuvant vaccines. *Br. abortus* 544 was the least effective in overcoming the immunity of vaccinated guinea pigs, although the $ID_{50}$ of this culture in non-vaccinated guinea pigs was of the same order as that of strains H38 and 6015. On the other hand, the $ID_{50}$ of strain 177 in non-vaccinated guinea pigs was significantly higher than that of H38, which would suggest that it was a less virulent strain for guinea pigs. It would appear that the $ID_{50}$ of a strain, as determined in normal guinea pigs, is not necessarily correlated with its ability to overcome the immunity produced by a vaccine.

## CONCLUSIONS

Studies in guinea pigs have shown that the immunity produced by the inoculation of a living avirulent *Br. melitensis* culture was significantly greater than that produced by killed rough and smooth cultures in adjuvant. Challenge with four different strains of brucella did not reveal significant differences in the protection afforded by the rough and smooth adjuvant vaccines.

## ACKNOWLEDGMENTS

We express our sincere thanks to Dr. A. W. Stableforth for his advice and interest, and to Dr. Irma G. Regueiro of Inspeccion General de Veterinaria, Madrid, for valuable help during her stay at Weybridge. Dr. Elberg's cooperation in providing the living vaccine is gratefully acknowledged.

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[*Received for publication, January 16th,* 1958]

# THE HISTOPATHOLOGY OF THE BRAIN STEM OF SHEEP AFFECTED WITH EXPERIMENTAL SCRAPIE

By

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## INTRODUCTION

Cuillé and Chelle (1936, 1938), Greig (1950) and Gordon (1946) have furnished evidence that scrapie can be experimentally transmitted from sheep to sheep. Further work by Wilson, Anderson and Smith (1950) has shown that the disease is transmissible in series by intracerebral and subcutaneous inoculations of scrapie brain suspensions. The clinical disease thus produced did not differ from that occurring naturally in sheep, but the histopathological picture was not entirely similar to that described by Holman and Pattison (1943) for natural scrapie. According to Wilson *et al.* (1950), in the experimental disease vacuolation of neurones of the medulla was slight and variable and lesions were present which were indicative of subacute or chronic meningo-encephalitis. These findings appear to be rather conflicting especially in view of the work of Pattison (1957) who reported severe vacuolation of neurones in the medulla of goats experimentally infected with scrapie and of the studies in the pathology of natural scrapie by Wagner, Goldstein, Doran and Hay (1954), Palmer (1957) and Zlotnik (1958) who stressed the non-inflammatory character of the lesions in the brain.

In the present paper, apart from the study of the histological changes in experimental scrapie, an attempt is also made to ascertain whether any specific changes occur in the brain stem of inoculated animals before the onset of the clinical disease.

## MATERIALS AND METHODS

All the sheep used in the present study were of the Cheviot breed and formed a part of large scale transmission experiments carried out at the Moredun Institute. The actual material for the investigation consisted of brain stems from 62 sheep divided into the following groups.

*Experimental animals exhibiting clinical symptoms of scrapie.* Twenty-one positive cases were chosen from larger groups of sheep inoculated with scrapie brain suspension and showing definite clinical symptoms of scrapie. The incubation period in these animals ranged from $4\frac{1}{2}$ to $6\frac{1}{2}$ months while the clinical course, from the onset of symptoms till destruction, varied from 1 to 9 weeks. The sheep were subdivided as follows:— (*a*) six lambs, $4\frac{1}{2}$ to 6 months old, inoculated intracerebrally at birth or very soon thereafter; (*b*) ten hoggs, 1 to $1\frac{1}{4}$ years old, inoculated intracerebrally at the age of 6 to 8 months; (*c*) five sheep inoculated subcutaneously; three of these animals were inoculated at the age of about 8 months while two were 2 years old.

*Experimental animals destroyed prior to developing clinical symptoms of scrapie.* Twenty hoggs, 6 to 8 months old, were inoculated intracerebrally and divided into 2 groups of 10 animals each as follows:— (*a*) ten animals to be destroyed 2 months after inoculation; (*b*) ten animals to be destroyed

4 months after inoculation. None of the above animals exhibited any clinical symptoms of scrapie at the time of their destruction.

*Controls.* Twenty-one apparently healthy uninoculated sheep were chosen from larger consignments on the basis of their ages only, in order to compare the histology of their brain stem with that of the experimental clinical cases. Like the experimental sheep these controls were divided into 3 groups as follows:— (*a*) six 6-month old lambs; (*b*) ten 1-year old hoggs; (*c*) five over 1-year old (2 sheep about $2\frac{1}{2}$ years old and 3 hoggs about $1\frac{1}{4}$ years old).

*Inoculum.* The material for both intracerebral and subcutaneous inoculations consisted of pooled brains obtained from consecutive serial passages of scrapie brain in sheep and originating from the successful transmission of scrapie in sheep by Wilson *et al.* (1950). The brain tissue was triturated very finely and then suspended in saline in a $10^{-1}$ concentration. After one-hour centrifugation at approximately 3000 r.p.m. the supernatant fluid was collected and used for injecting in doses of 1 ml. for the intracerebral route and 5 ml. for subcutaneous administration.

*Post-mortem methods.* All the animals investigated in the present study were destroyed by exsanguination and the brains, removed immediately after death, were fixed in 10 per cent formol-saline for about 10 days. The technique of preparation of brain stems for histological examination and the method of counting vacuolated neurones was the same as described previously for natural scrapie (Zlotnik, 1958).

## RESULTS

### *Experimental Animals exhibiting Clinical Symptoms*

Vacuoles were present in each of the 3 blocks of the medullas from all the 21 animals (Table 1). In the lambs inoculated at birth the number of vacuoles was somewhat higher than in the other 2 groups, the average range per section being 2·8 to 14·8 vacuoles. On the other hand no appreciable difference could be found in the incidence of vacuoles between sheep inoculated subcutaneously and those injected intracerebrally, the average range in the former group being 3·4 to 7·7 vacuoles per section and in the latter 2·2 to 7·5 vacuoles. The part of the medulla most severely affected by vacuolation varied from block "B" in the lambs and the subcutaneous group to block "A" in the 1-year old hoggs. As a whole in a total of 1134 sections from medullas of 21 sheep, 6445 vacuoles were found: the distribution was 28 per cent in block "A", 38 per cent in block "B" and 34 per cent in block "C".

Apart from the medulla, vacuoles were invariably found in the pons and in most cases in the mesencephalon (Table 2). In the 15 brain stems, where the mesencephalon has been studied, vacuoles were present in all but 2 cases. The figures given in Table 2 for the mesencephalon relate to the following 3 nuclei only—red nucleus, motor-tegmentopeduncular nucleus and pigmented cells. The number of vacuoles varied from 0·1 to 6·9 per section. In the pons vacuolation proved to be similar in extent to that of the medulla, the average number of vacuoles per section ranging from 1·0 to 28·3.

A comparison of the distribution of vacuolated neurones in the pons and the medulla showed that in a total of 15 cases the average number of vacuoles per section was higher in the pons than in the medulla in 8 sheep, while in 7 cases only the pons contained less vacuoles than the medulla.

TABLE 1

VACUOLATION AND DEGENERATION OF NEURONES IN THE MEDULLA IN EXPERIMENTAL CLINICAL SCRAPIE

| *Serial no.* | *Total no. of vacuoles in 18 consecutive sections in parts of the medulla* | | | *No. of vacuoles in 54 sections of the medulla* | | *Neurone changes* | | |
|---|---|---|---|---|---|---|---|---|
| | *Medulla Block "A"* | *Medulla Block "B"* | *Medulla Block "C"* | *Total* | *Average per section* | *Chromatolysis* | *Pyknosis* | *Necrosis* |
| I | Lambs inoculated intracerebrally at birth | | | | | | | |
| Exp. S. 36 | 90 | 189 | 72 | 351 | 6·5 | ++ | ++ | + |
| ,, ,, 37 | 138 | 36 | 30 | 204 | 3·8 | — | +++ | — |
| ,, ,, 38 | 57 | 300 | 291 | 648 | 12·0 | + | +++ | — |
| ,, ,, 39 | 30 | 63 | 57 | 150 | 2·8 | + | +++ | + |
| ,, ,, 143 | 19 | 210 | 108 | 337 | 6·2 | + | — | — |
| ,, ,, 144 | 60 | 378 | 360 | 798 | 14·8 | +++ | — | — |
| Total in 6 lambs | 394 | 1176 | 918 | 2488 | | | | |
| II | Sheep inoculated intracerebrally at the age of 6–8 months | | | | | | | |
| Exp. 61 | 99 | 36 | 272 | 407 | 7·5 | + | — | + |
| ,, 111 | 90 | 51 | 37 | 178 | 3·3 | ++ | — | + |
| ,, 134 | 168 | 69 | 45 | 282 | 5·2 | +++ | ++ | — |
| ,, 150 | 98 | 52 | 54 | 204 | 3·8 | ++ | ++ | — |
| ,, 155 | 99 | 24 | 40 | 163 | 3·0 | ++ | +++ | — |
| ,, 156 | 75 | 138 | 45 | 258 | 4·8 | ++ | ++ | + |
| ,, 157 | 210 | 120 | 75 | 405 | 7·5 | +++ | ++ | ++ |
| ,, 158 | 48 | 39 | 33 | 120 | 2·2 | +++ | — | — |
| ,, 160 | 48 | 51 | 210 | 309 | 5·7 | +++ | ++ | ++ |
| ,, 166 | 72 | 144 | 39 | 255 | 4·7 | +++ | +++ | ++ |
| Total in 10 hoggs | 1007 | 724 | 850 | 2581 | | | | |
| III | Sheep inoculated subcutaneously | | | | | | | |
| Exp. 88 | 42 | 258 | 48 | 348 | 6·4 | +++ | ++ | ++ |
| ,, 154 | 76 | 78 | 66 | 220 | 4·1 | +++ | — | — |
| ,, D. 71 | 78 | 105 | 21 | 204 | 3·8 | +++ | — | ++ |
| ,, C. 245 | 186 | 75 | 156 | 417 | 7·7 | ++++ | +++ | +++ |
| ,, C. 260 | 18 | 46 | 123 | 187 | 3·4 | +++ | — | + |
| Total in 5 sheep | 400 | 562 | 414 | 1376 | | | | |
| Total in 21 sheep | 1801 | 2462 | 2182 | 6445 | | | | |

Exp. 88, 154 and D. 71—inoculated at the age of 8 months.
Exp. C. 245 and 260—inoculated at the age of 2 years.

\+ occasional
++ few
+++ fair nos.
++++ numerous

While the vacuoles occurring in the natural disease were of many types, in the experimental animals vacuoles as a whole were less complex. Approximately 90 per cent were single cavities present either in otherwise unaltered cytoplasm, in pyknotic cells, in sclerotic neurones or in chromatolytic cells. About 5 per cent of single vacuoles caused extreme ballooning of the cytoplasm and necrobiotic changes in the nucleus. The multiple foamy vacuoles which were very frequently present in the natural disease were more rarely seen in experimental scrapie, forming only about 5 per cent of the total number of vacuoles. In a proportion of vacuoles eosinophilic bodies were present, similar to those described for healthy sheep and for sheep affected with natural scrapie.

TABLE 2

VACUOLATION OF NEURONES IN THE BRAIN STEM IN EXPERIMENTAL CLINICAL SCRAPIE

Average Number of Vacuoles per Section

| *Serial no.* | *Mesencephalon* | *Pons* | *Medulla* |
|---|---|---|---|
| Exp. S. 36 | 6·9 | 6·9 | 6·5 |
| ,, ,, 37 | 0·1 | 8·8 | 3·8 |
| ,, ,, 38 | Nil | 9·6 | 12·0 |
| ,, ,, 39 | 0·1 | 2·16 | 2·8 |
| ,, D. 71 | 0·3 | 5·0 | 3·8 |
| ,, C. 245 | Nil | 1·5 | 7·7 |
| ,, S. 143 | 0·4 | 1·7 | 6·2 |
| ,, ,, 144 | 1·5 | 8·3 | 14·8 |
| ,, 150 | 2·5 | 8·2 | 3·8 |
| ,, 154 | 1·0 | 1·0 | 4·07 |
| ,, 155 | 0·2 | 12·1 | 3·0 |
| ,, 156 | 0·1 | 5·16 | 4·8 |
| ,, 157 | 1·7 | 28·3 | 7·5 |
| ,, 158 | 2·2 | 7·0 | 2·2 |
| ,, 160 | 0·2 | 5·0 | 5·7 |

Apart from vacuolation, various degrees of degeneration of neurones, ranging from partial or total chromatolysis, pyknosis and sclerosis of neurones to complete necrosis, were noted in all the experimental animals. Degeneration affected either scattered neurones of the brain stem or whole nuclei. When whole nuclei were undergoing degeneration it was found that the process had a rather symmetrical distribution, although not always did the two nuclei on both sides of the stem show the same degree of changes. In the mesencephalon the large neurons of the red nucleus were found to undergo chromatolysis in 75 per cent of animals examined. In the pons degeneration was scattered, but the lateral vestibular nucleus and the reticular formation were most often affected. In the trapezoid body of the medulla the neurones of the lateral and medial vestibular nuclei were nearly always undergoing chromatolysis, while lower down the cells of the raphé and of the reticular

formation, especially in the formatio reticularis alba, were showing various degrees of chromatolysis and pyknosis (Table 1).

A study of glial elements did not reveal any evidence of astrocytic proliferation or abnormal distribution. Focal microglial accumulations were seen in 43 per cent of animals while neuronophagia was observed in 57 per cent of cases. Perivascular and nodular infiltrations of lymphocytes and plasma cells were observed in about 70 per cent of animals. These however were very slight and did not differ from changes seen in apparently healthy sheep (Zlotnik and Rennie, 1957). No myelin changes of any significance could be detected in any of the experimental animals.

*Experimental Animals Destroyed prior to developing Clinical Symptoms*

*Sheep destroyed 2 months after inoculation.* Vacuolated neurones were found in 6 medullas only with an incidence ranging from 2 to 27 vacuoles in 54 sections (0·037 to 0·5 vacuoles per section). A total of 76 vacuoles were counted in 540 sections from 10 medullas. Although vacuoles were present in block "A" from 2 animals only, they formed 35 per cent of the total number of vacuoles in all the medullas. This distribution was due to the fact that in one case only, 25 vacuoles were present in block "A" (Table 3). All the vacuoles observed in the above animals appeared in the form of single cavities in otherwise normal neurones. Apart from vacuolation no other neuronal abnormality was observed, except that in one medulla an occasional necrotic cell could be seen. In two animals focal proliferation of microglia was noticeable. Perivascular changes, although noted in 9 cases, could only be classed as marked in two sheep.

*Sheep destroyed 4 months after inoculation.* Vacuolated neurones were present in 9 medullas (Table 4). The total number of vacuoles was 638 in 540 sections of the 10 medullas, an increase of 562 vacuoles over the group of animals destroyed 2 months after inoculation. All the 3 blocks of the medulla showed increased numbers and block "A" although containing less vacuoles than the other 2 blocks had a total of 158. This increase in vacuolation in animals destroyed 4 months after scrapie inoculation was due to a rise in counts in 3 animals only (Exp. 32, 36 and 38), while in the remainder no change could be noticed. In these 3 animals, 2 had vacuole counts almost averaging 2 vacuoles per section, while in the third there was an average of 6·6 vacuoles per section, a count similar to that of the experimental groups.

The majority of vacuoles observed in the medullas of this group were single cavities in otherwise unchanged cytoplasm, although some vacuolated neurones in the medullas from the 3 animals with increased counts were undergoing chromatolysis or pyknosis and a few were of the multiple type. Degenerative changes in unvacuolated neurones were also present and in 2 cases occasional necrotic cells could be found (Table 3). Neuronophagia appeared in two animals,

while small foci of microglial cells were found in 5 cases. Perivascular infiltration was observed in 7 cases, but in none could it be classed as severe.

TABLE 3

VACUOLATION AND DEGENERATION OF NEURONES IN THE MEDULLA OF EXPERIMENTAL SHEEP DESTROYED PRIOR TO DEVELOPING CLINICAL SYMPTOMS OF SCRAPIE

| *Serial no.* | *Total no. of vacuoles in 18 consecutive sections in parts of the medulla* | | | *No. of vacuoles in 54 sections of the medulla* | | *Neurone changes* | | |
|---|---|---|---|---|---|---|---|---|
| | *Medulla Block "A"* | *Medulla Block "B"* | *Medulla Block "C"* | *Total* | *Average per section* | *Chromatolysis* | *Pyknosis* | *Necrosis* |
| I | Sheep destroyed 2 months after inoculation | | | | | | | |
| Exp. 21 | — | — | 2 | 2 | 0·037 | — | — | + |
| „ 22 | — | 8 | — | 8 | 0·15 | — | — | — |
| „ 23 | — | — | — | Nil | — | — | — | — |
| „ 24 | — | — | — | Nil | — | — | — | — |
| „ 25 | — | — | 3 | 3 | 0·05 | — | — | — |
| „ 26 | — | — | — | Nil | — | — | — | — |
| „ 27 | — | — | 20 | 20 | 0·37 | — | — | — |
| „ 28 | 25 | — | 2 | 27 | 0·5 | — | — | — |
| „ 29 | — | — | — | Nil | — | — | — | — |
| „ 52 | 2 | 14 | — | 16 | 0·3 | — | — | — |
| Total in 10 sheep | 27 | 22 | 27 | 76 | | | | |
| II | Sheep destroyed 4 months after inoculation | | | | | | | |
| Exp. 30 | — | — | 3 | 3 | 0·05 | — | — | — |
| „ 31 | 2 | 3 | 3 | 8 | 0·15 | + | — | — |
| „ 32 | 21 | 60 | 25 | 106 | 1·96 | + | ++ | — |
| „ 33 | 6 | — | 21 | 27 | 0·5 | — | — | — |
| „ 34 | 3 | — | 3 | 6 | 0·1 | — | — | — |
| „ 35 | — | — | — | Nil | — | — | — | — |
| „ 36 | 51 | 12 | 42 | 105 | 1·94 | ++ | ++ | + |
| „ 37 | 3 | 9 | 5 | 17 | 0·3 | — | — | — |
| „ 38 | 72 | 171 | 117 | 360 | 6·6 | ++ | ++ | + |
| „ 39 | — | 6 | — | 6 | 0·1 | — | — | — |
| Total in 10 sheep | 158 | 261 | 219 | 638 | | | | |

*Controls*

Amongst the 21 brain stems from apparently healthy sheep, vacuolated neurones have been found in 18 medullas (85·7 per cent). There was no essential difference in the incidence of vacuoles between the 3 sub-groups and the number varied from 3 to 61 in 54 sections of the medulla (0·05 to 1·13 per section). No vacuoles were observed in the pons, mesencephalon or medulla-cord junction. In one medulla the number was as high as 61 and in 2 animals there was more than one per 2 sections. The vacuoles as a whole were never evenly distributed throughout the medulla, the least affected part being the trapezoid body (block "A") where vacuoles were

found in 3 cases only, while the posterior medulla (block "C") contained the highest number (Table 4). The total count in 1134 sections from 21 medullas was 323 with 6 only present in block "A", and the highest count of 184 in block "C". Vacuolated neurones were found in the medulla in the following nuclei: dorsal motor vagus, paramedianus dorsalis, reticular formation, medial vestibular, facial and arcuate. The dorsal motor vagus, however, usually contained more vacuoles than any other nucleus (Table 4).

TABLE 4

VACUOLATION AND DEGENERATION OF NEURONES IN THE MEDULLA OF APPARENTLY HEALTHY SHEEP

| *Serial no.* | *Total no. of vacuoles in 18 consecutive sections in parts of the medulla* | | | *No. of vacuoles in 54 sections of the medulla* | | *Neurone changes* | | |
|---|---|---|---|---|---|---|---|---|
| | *Medulla Block "A"* | *Medulla Block "B"* | *Medulla Block "C"* | *Total* | *Average per section* | *Chromatolysis* | *Pyknosis* | *Necrosis* |
| I | Six months-old lambs | | | | | | | |
| N. 11 | 1 | 2 | 2 | 5 | 0·09 | — | — | — |
| „ 12 | — | 19 | 9 | 28 | 0·51 | — | — | — |
| „ 13 | — | 11 | 4 | 15 | 0·27 | — | — | — |
| „ 14 | — | 2 | 19 | 21 | 0·39 | — | — | — |
| „ 15 | — | — | 13 | 13 | 0·24 | — | — | — |
| „ 16 | — | 5 | 9 | 14 | 0·26 | — | — | — |
| Total in 6 lambs | 1 | 39 | 56 | 96 | | | | |
| II | One year-old hoggs | | | | | | | |
| N. 1 | — | 19 | 3 | 22 | 0·4 | — | — | — |
| „ 2 | — | — | — | Nil | — | — | — | — |
| „ 3 | — | 29 | 6 | 35 | 0·65 | — | + | + |
| „ 4 | — | — | 16 | 16 | 0·29 | — | — | — |
| „ 5 | — | — | — | Nil | — | — | — | — |
| „ 6 | 3 | — | — | 3 | 0·05 | — | — | — |
| „ 7 | — | 7 | 10 | 17 | 0·31 | — | — | — |
| „ 8 | — | 4 | 11 | 15 | 0·27 | — | — | — |
| „ 9 | — | 3 | 4 | 7 | 0·13 | — | — | — |
| „ 10 | — | 6 | 7 | 13 | 0·24 | — | — | — |
| Total in 10 hoggs | 3 | 68 | 57 | 128 | | | | |
| III | Over one year-old sheep | | | | | | | |
| N. 17 | — | 8 | 9 | 17 | 0·31 | — | — | — |
| „ 18 | — | 18 | 43 | 61 | 1·13 | — | — | — |
| „ 19 | 2 | — | 15 | 17 | 0·31 | — | — | — |
| „ 20 | — | — | — | Nil | — | — | + | — |
| „ 21 | — | — | 4 | 4 | 0·07 | — | — | — |
| Total in 5 sheep | 2 | 26 | 71 | 99 | | | | |
| Total in 21 sheep | 6 | 133 | 184 | 323 | | | | |

N. 17–19—1¼ years old
N. 20–21—2½ years old

Apart from vacuolation, other neuronal changes were very seldom observed. In two cases only a very few degenerating pyknotic cells were encountered and in one medulla an occasional necrotic cell was seen. The glial and vascular changes were similar to those already described in a previous paper (Zlotnik and Rennie, 1957).

## DISCUSSION

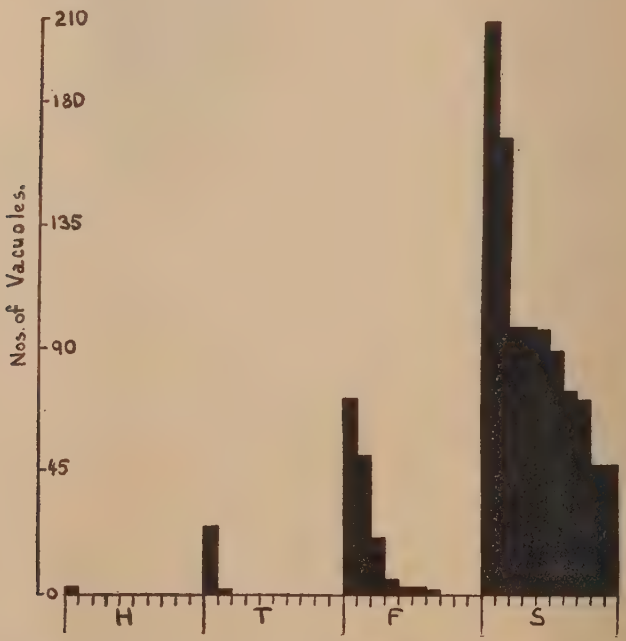
The results of the present study show that in experimental scrapie vacuolation and degeneration of neurones, with variable degrees of glial involvement, form the essential histological changes in the brain stem. In this respect the findings are similar to those for natural scrapie (Wagner *et al.*, 1954; Zlotnik, 1958). The claim however of Wilson *et al.* (1950) that the histological picture is that of a subacute or chronic meningo-encephalitis could not be confirmed, as no inflammatory lesions were found in any of the brain stems examined that could not be found in normal sheep. On the other hand their observation that vacuolation of neurones in the medulla of experimentally infected sheep was less severe than in those of naturally occurring scrapie cases was supported by the present work.

Throughout the present paper the incidence of vacuolated neurones in parts of the brain stem was studied by means of detailed counts in serial sections. This procedure was necessary in view of the fact that vacuoles were reported in neurones of the medulla from healthy sheep (Zlotnik and Rennie, 1957). Careful counting of vacuoles in the experimental disease showed that in 54 sections of the medulla from 21 animals a range of 120 to 798 vacuoles was found, while in 20 animals affected with the natural disease the numbers were higher giving a range of 435 to 5832 (Zlotnik, 1958). The fact that vacuoles are fewer in the experimental disease does not, however, alter their significance since serial section counts show that the number of vacuoles in the brains of sheep affected with experimental scrapie are still much higher than in the brain of healthy sheep. A study of the incidence of vacuolated neurones in lambs and hoggs inoculated intracerebrally, and older animals inoculated subcutaneously gives further evidence that increased vacuolation in experimental scrapie occurs irrespective of age of the animal or route of inoculation. It would appear, therefore, that vacuolation of neurones is indicative of, if not actually specific for, that disease.

The method of detailed counts of vacuolated neurones in the medulla proved that not only in experimental clinical scrapie was there a significant increase in vacuoles, but also that this occurred before any clinical symptoms could be detected. In animals destroyed 2 months after inoculation with scrapie, although the total counts were more or less similar to those in healthy sheep, in one animal a count of 25 vacuoles in 18 sections was found in the trapezoid body (block "A"). This increase in vacuolation is unusual for the trapezoid body and may suggest a reaction to the inoculum, since evidence

based on examination of 91 healthy sheep of various breeds indicates that such a frequency of vacuoles is not present in the non-scrapie animal (Zlotnik, 1958; Zlotnik and Rennie, 1958). The counts recorded hitherto for healthy sheep, range from 0·05 to 0·3 vacuoles per section and then only in 32 per cent of animals examined. In medullas of animals destroyed 4 months after inoculation, the incidence of vacuoles in 7 sheep was within the range of healthy sheep, while in the remaining 3 a definite rise in vacuolation similar to that in experimental clinical cases was obvious with corresponding increases in the trapezoid body. Since this incidence of vacuolation is similar to the number of takes after artificial transmission of scrapie (Wilson *et al.* 1950) it may be that these 3 animals were in the incubation period of scrapie. A comparison of incidence of vacuolated neurones in the trapezoid body in healthy animals, preclinical cases and clinical scrapie, shows that while in healthy sheep vacuolation is very slight, in the clinical disease it is a constant feature and therefore may prove of value in assessing preclinical infections (Text Fig. 1).

Fig. 1




A comparison of the incidence of vacuolated neurones in the trapezoid body between 4 groups of one year-old hoggs.

H = apparently healthy sheep.
T = experimental sheep destroyed 2 months after inoculation.
F = experimental sheep destroyed 4 months after inoculation.
S = experimental clinical scrapie.

Various degrees of neurone degeneration throughout the brain stem were recorded in the present study, so that in this respect the findings are very similar to those of animals affected with the naturally occurring disease except that completely necrotic cells were more rarely observed. Degenerated neurones in the experimental disease which showed a greater tendency to be grouped together in certain nuclei were present not only in the sheep showing clinical signs but also in the 3 animals which showed increased vacuolation 4 months after inoculation. No significant degenerative changes were present in animals destroyed 2 months after inoculation with scrapie brain suspension, so that if the increase in vacuolation in the trapezoid body, recorded in one such animal, can be considered a reaction to the inoculum, vacuolation of neurones may be the primary lesion preceding any other neuronal or glial change.

The vascular changes found in the brain stem of experimental animals were as a whole very similar in type, severity and distribution to these present in apparently healthy sheep and, therefore, have no special significance in scrapie. The glial changes found in the experimental disease differed in some respects from those recorded in the natural disease. The astrocytic proliferation found in natural scrapie was not observed in the experimental disease where the glial proliferation involved oligodendroglial and microglial cells only. The perivascular demyelination present in advanced natural scrapie was almost completely absent in experimentally induced scrapie.

## CONCLUSIONS

Vacuolation and degeneration of neurones is a constant lesion in experimental scrapie.

Vacuolation of neurones in the medulla precedes the clinical disease.

The trapezoid body, where vacuoles are rarely observed in healthy animals, is always affected in the experimental disease.

In experimental scrapie vacuolation of the neurones of the pons is at least as severe as that of the medulla.

The brain stem lesions in experimental scrapie are similar in animals inoculated intracerebrally and in sheep injected subcutaneously.

## ACKNOWLEDGMENTS

The author wishes to thank Dr. J. T. Stamp, Director of the Moredun Institute, for the interest he has taken in this work and for his useful advice and suggestions. Special thanks are due to Mr. J. C. Rennie for his technical assistance. The writer records his appreciation to Mr. W. Smith for the preparation of scrapie brain suspensions and for carrying out the scrapie inoculations.

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[*Received for publication, January 17th*, 1958]

# EXPERIMENTAL TRANSMISSION OF AVIAN OSTEOPETROSIS

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## INTRODUCTION

Avian osteopetrosis, also known as Thick Leg Disease, is a sporadic disease characterised by a progressive thickening affecting primarily the diaphyses of the long bones. It is generally bilaterally symmetrical. The condition usually becomes clinically evident at about 3 months of age, when visible thickening of the metatarsi occurs which may become so extensive that the legs assume a boot-like appearance. The incidence is generally low.

Jungherr and Landauer (1938) observed only 5 cases during the routine post mortem examination of 10,000 birds over six weeks of age and Jordan (1956) during the examination of 4,459 carcases encountered only two cases. The condition has been described by some workers as Osteopetrotic Lymphomatosis because of its apparent association in some cases, with the lesions of lymphomatosis. Further weight may have been added to this opinion by the fact that in experiments on the transmission of lymphomatosis reported by American workers osteopetrosis has followed the inoculation of chicks with cellular and cell-free suspensions of both blood and tumour tissue from donor birds with neural or diffuse visceral lymphomatosis. Jungherr and Landauer (1938) successfully transmitted the disease with cellular suspensions of whole blood, bone marrow and tumour material using active cases of osteopetrosis as donor birds. Brandley, Nelson and Cottral (1942) set up osteopetrosis following inoculation into chicks of whole blood from donors with no evidence of osteopetrosis, but suffering from iritis and neurolymphomatosis. Burmester, Prickett and Belding (1946), Burmester (1947) and Burmester and Cottral (1947) found that centrifuged extracts of lymphoid tumour cells or filtered plasma from birds with lymphoid tumours injected into 2 to 3 day old chicks induced in 6 months a high incidence of osteopetrosis as well as visceral lymphomatosis.

There is no definite evidence at present, however, that a common etiological agent is concerned: in fact Burmester and Cottral (1947) and Burmester and Gentry (1954) concluded from the results of their transmission experiments that osteopetrosis, visceral lymphomatosis and neural lymphomatosis are produced by different agents and that either may remain latent in recipients and then reappear in subsequent passages.

This investigation, which was undertaken to study the transmissibility of avian osteopetrosis in Great Britain, was concerned with the most suitable materials to use, the most effective routes of administration and the possible modes of natural transmission.

## MATERIALS AND METHODS

The materials used for transmission were whole blood, plasma and bone marrow. Blood was obtained by cardiac puncture and collected

in heparin. In day old chicks it was injected undiluted intraperitoneally immediately after collection except in one experiment in which it was stored for 24 hours at 4°C prior to use. Plasma was obtained by centrifuging freshly collected blood at 2,000 r.p.m. for 5 minutes. The plasma was then immediately pipetted off and 0·5 ml. injected intraperitoneally. Bone marrow was obtained from Donor No. 1 immediately after death. The right tibia was sliced longitudinally and the marrow removed into sterile normal saline. This was shaken, the gross sediment allowed to settle and the supernatant was injected intraperitoneally or into the lower end of the right tibia of day old chicks. In the former case 0·5 ml. of the suspension was used and in the latter 0·2 ml.

Intra-amnion inoculation of chick embryos was carried out by the method described by Lépine and Sohier (1954) using variation III of the direct vision technique (Sohier and Esser-Trimberger, 1952). The embryos were usually from 11 to 13 days of age when inoculated with 0·05 to 0·1 cc. of whole or diluted blood or bone marrow which had been stored for from 24 to 72 hours at 4°C prior to use. Blood was diluted with normal saline immediately before injection.

Day old chicks were obtained from a commercial hatchery and except in one experiment, where only pullets were used, they were "as hatched". A wide selection of breeds and crosses was used, randomised as far as possible in each batch. These included R.I.R., L.S., Br.L., N.H.B., R.I.R. x L.S., L.S. x R.I.R., Br.L. x R.I.R. and Br.L. x L.S. Although in the field the incidence of osteopetrosis is low the possibility of the introduction of naturally infected chicks into the experiments had to be considered. In each experiment some birds were therefore left uninoculated to serve as controls.

Eggs for amnion inoculation, with a few exceptions which were produced at the laboratory, were obtained from a commercial hatchery as 10 day old embryos. These were all R.I.R. x L.S. Some were left as controls and were not inoculated.

The chicks in the control and injected groups were reared in separate airspaces under Infra-red lamps up to about 5 weeks old and were put out in fold arks on grass at 7 to 8 weeks of age. The birds were examined every 14 days from the time of hatching and the limbs palpated for any signs of bony enlargement. If this was detected they were then X-rayed using Kodak Kodirex X-ray film. All birds which died during the experiment were examined post mortem and the legs and wings were X-rayed. Both control and injected birds were kept for at least 6 months and in some batches for up to 7½ months before slaughter. A thorough post mortem examination was then carried out and the legs and wings of each bird were X-rayed. Ilford Industrial F X-ray film was used for post mortem material and skin and muscles were removed prior to radiography.

The initial donor (female Br.L. x R.I.R.) was a field case of the disease with the following history. A batch of 100 Br.L. x R.I.R. day old chicks was purchased by a poultry farmer in March, 1956. Mortality was very low and 97 birds were removed from the brooder to field arks. Three cases of osteopetrosis appeared, thickening of the metatarsi of these birds being first noticed at about 3 months of age. The owner had never previously seen the condition. Egg production was good and no further cases developed.

The donor (No. 1) was examined radiologically every 14 days from 5th October until first used for whole blood transmission on 25th January, 1957. Clinically there was increased heat in the metatarsal region, palpable thickening of the other limb and wing bones and radiological evidence of very active new bone formation as shown by the series of radiographs in Fig. 1. On 5th February, 1957 this bird was bled out and whole blood and bone marrow obtained for transmission experiments. Post mortem examination revealed no gross abnormalities in the viscera but marked changes in the bones, typical of avian osteopetrosis.

## RESULTS

The results of using whole blood or bone marrow from the original donor (No. 1) clearly indicated that the disease was transmissible. A total of 60 day old chicks was inoculated and 24 developed osteopetrosis. In the second passage five donors were used (Table 1) for the inoculation of 35 day old chicks of which 11 developed the disease. These results are illustrated in Fig. 7.

Fig. 7

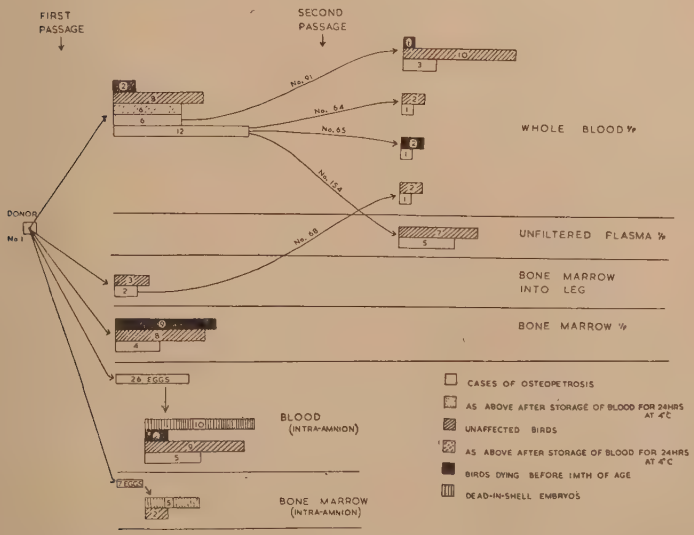


Chart of transmission experiments. The numbers in the blocks indicate the numbers of chicks concerned.

### *Intraperitoneal Injection of Day Old Chicks*

In the first experiment using whole heparinised blood from No. 1 four different doses were given (Table 2). Although the number of birds used was small the results show that a dose of 0·25 ml. was effective in transmitting the disease. After storage of blood a dose of 0·5 or 0·75 ml. also induced osteopetrosis. Following

these results it was decided to use a standard dose of 0.5 ml. by intraperitoneal injection in subsequent experiments.

TABLE 1

DETAILS OF DONOR BIRDS USED IN THE SECOND PASSAGE

| *No.* | *Breed* | *Sex* | *Method of infection** | *1st symptoms noted (days)* | *Visible thickening of metatarsi (days)* | *Bled for transmission (days)* | *Fig. No.* |
|---|---|---|---|---|---|---|---|
| 91 | R.I.R. x L.S. | ♂ | 0·75 cc. whole blood I/P kept for 24 hrs. at 4°C before inoculation | 49 | 60 | 51 | 2 |
| 64 | Br.L. x R.I.R. | ♀ | 0·5 cc. whole blood I/P | 50 | 60 | 55 | 3 |
| 65 | Br.L. x L.S. | ♀ | 0·5 cc. whole blood I/P | 50 | — | 55 | 5 |
| 68 | Br.L. x R.I.R. | ♀ | 0·2 cc. bone marrow into Rt. tibia | 50 | 92 V. marked | 55 | 4 |
| 154 | R.I.R. x L.S. | ♂ | 0·5 cc. whole blood I/P | 64 | 106 | 132 | 6 |

* From Donor No. 1. All injected at 1 day old.

*Effect of Route of Administration*

Injections were made intraperitoneally or into the lower end of the right tibia. The results of intraperitoneal injection are shown in Table 3. The tibial route was used firstly to determine if the disease could be transmitted by introducing a small amount of inoculum into the distal tibial epiphysis and secondly to see if the lesion resulting would be confined to the injected bone and if not to study the symmetry of the lesions. Bone marrow alone was used and the maximum amount which could be injected with ease at this site was 0·2 ml. Of 5 chicks inoculated two developed the disease and the resulting lesions were bilaterally symmetrical.

TABLE 2

THE EFFECT OF VARYING DOSES OF WHOLE BLOOD FROM DONOR NO. 1. INJECTED INTRAPERITONEALLY INTO DAY-OLD CHICKS

| *Dose* | *Total No. injected* | *Cases of osteopetrosis in birds surviving 1 month* | | |
|---|---|---|---|---|
| ml. | | +ve | —ve | |
| 0·25 | 2 | 1 | 1 | Fresh blood |
| 0·5 | 16 | 10 | 4 | |
| 0·75 | 2 | 1 | 1 | |
| 1·0 | 2 | 0 | 2 | |
| 0·5 | 6 | 2 | 4 | After storage at 4°C for 24 hours |
| 0·75 | 6 | 4 | 2 | |

*Infectivity of Various Materials*

It is evident that whole blood was infective both when injected immediately after collection from the donor and after storage for 24 hours at 4°C. (Table 3). The disease was also reproduced by bone marrow and unfiltered plasma. Although a greater number of chicks was injected with whole blood, the results suggest that the infectivity of the three materials used was very similar. Nevertheless, the bone marrow suspension caused a high early mortality, 9 birds dying during the first 10 days of life, 8 from *E. coli* peritonitis and one from rupture of the yolk sac. As a saw was used to split the dense tibial shaft and bone dust was probably introduced with the marrow tissue, it was difficult to maintain the material in an aseptic condition.

*Intra-amnion Inoculation*

To determine whether the egg might act as one means of natural transmission developing eggs incubated from 8 to 13 days were inoculated into the amniotic cavity with whole and diluted blood

and bone marrow suspension. The results are shown in Table 4. From 31 eggs inoculated 18 chicks were hatched and 16 were alive at 1 month old. Thirteen embryos died between the 10th and 20th day of incubation. Only 2 embryos appeared to have been infected as a result of the injection. Twelve chicks were hatched from 16 control eggs from the same batch and all survived to 7 months of age.

The results indicate that it is possible at least experimentally for the transmissible agent to pass from the amnion to the developing embryo, for the embryo to survive and later develop typical osteopetrosis. Transmission was successfully accomplished using whole blood and blood diluted 1 in 10 and 1 in 100 with normal saline injected into embryos of 8 to 12 days incubation. In these experiments bone marrow did not produce any cases but the number of eggs used was small.

TABLE 3

EXPERIMENTS TO INDICATE THE INFECTIVITY OF VARIOUS MATERIALS BY INTRAPERITONEAL INJECTION

| *Material* | *Injected birds* | | | *Control birds* | | |
|---|---|---|---|---|---|---|
| | *Total no.* | *No. surviving 1 month* | | *Total no.* | *No. surviving 1 month* | |
| | | + | — | | + | — |
| Whole blood (Fresh) | 45 | 18 | 22 | 49 | 0 | 46 |
| Whole blood after 24 hrs. at 4°C | 12 | 6 | 6 | | | |
| Bone Marrow .. | 21 | 4 | 8 | | | |
| Unfiltered Plasma | 12 | 5 | 7 | 7 | 0 | 6 |
| Total.. .. .. | 90 | 33 | 43 | 56 | 0 | 52 |

*Sex Susceptibility*

In all the experiments males and females were approximately equal in numbers except in one case when only pullets were used. The latter batch has therefore been omitted in considering the possible relationship between sex and susceptibility to the disease. Table 5 shows that male birds were very significantly more susceptible than females. Clinical, post mortem and X-ray examination of the control birds revealed no cases of osteopetrosis or bone changes in any way resembling those in some of the experimentally infected birds.

*Age at First Symptoms*

Four cases characterised by palpable thickening of the mid shaft of the tibia, were detected as early as 35 days of age. The

TABLE 4

EFFECTS OF INTRA-AMNION INOCULATION OF CHICK EMBRYOS

| *Inoculum* | *Age of embryo (days)* | *Dead-in-shell* | | *No. of birds surviving 1 month* | *No. of cases of osteopetrosis* |
|---|---|---|---|---|---|
| | | *No.* | *Age at death (days)* | | |
| Whole blood .. | 8 | — | — | 1 | 1 |
| ,, ,, .. | 10 | 2+1* | 11+20 | — | — |
| ,, ,, .. | 11 | 1 | 20 | 3 | 2 |
| ,, ,, .. | 12 | 1 | 12 | 1 | — |
| ,, ,, .. | 13 | 1 | 10 | 1 | — |
| 1/5 Blood .. | 12 | 1 | 12′ | — | — |
| ,, ,, .. | 13 | 1* | — | 1 | — |
| 1/10 Blood .. | 11 | 1 | 20″ | 1 | — |
| ,, ,, .. | 12 | 1 | 12 | 2 | 1 |
| ,, ,, .. | 13 | 2 | 13+14′ | — | — |
| 1/100 Blood .. | 11 | — | — | 2 | 1 |
| ,, ,, .. | 12 | — | — | 1 | — |
| ,, ,, .. | 13 | — | — | 1 | — |
| Bone Marrow .. | 10 | 2 | 6+19″ | — | — |
| ,, ,, .. | 11 | 1 | 19 | 1 | — |
| ,, ,, .. | 12 | 1 | 12 | — | — |
| ,, ,, .. | 13 | 1 | 13 | 1 | — |
| Total .. .. | | 15+2* | | 16 | 5 |

*Died 3 days after hatching. 'Infected embryo.
″Embryo in lethal position.

TABLE 5

RESULTS OF THE TRANSMISSION EXPERIMENTS IN RELATION TO SEXES OF AFFECTED BIRDS

| *Inoculum* | *Birds surviving 1 month* | | *Cases of osteopetrosis* | |
|---|---|---|---|---|
| | ♂ | ♀ | ♂ | ♀ |
| Whole blood i/p .. .. .. | 22 | 23 | 13 | 5 |
| Bone Marrow i/p .. .. .. | 3 | 4 | 2 | 0 |
| Unfiltered plasma i/p .. .. | 6 | 6 | 4 | 1 |
| Blood intra-amnion .. .. | 9 | 7 | 5 | 0 |
| Totals .. | 40 | 40 | 24 | 6 |

60% of injected males +ve 15% of injected females +ve $X^2=15{\cdot}4$ $P<0{\cdot}001$

majority were recognised from approximately 50 to 100 days after inoculation but in three birds bone changes were not noted in life although they were detected by routine radiological examination of the long bones at post mortem. Following intra-amnion inoculation the earliest case was detected at 66 days of age.

## DISCUSSION

The observations reported in this paper confirm those of Jungherr and Landauer (1938). They indicate that a transmissible agent is involved in the etiology of avian osteopetrosis and that it is present in whole blood, bone marrow and unfiltered plasma. As some of the experimental cases are alive it is not possible, as yet, to determine whether there is any association between osteopetrosis and lymphomatosis, but it is hoped to report later on this subject.

In these experiments, of 97 birds surviving over 1 month which were infected either by inoculation into the amnion or by injection on the first day after hatching, 41 per cent. developed osteopetrosis. The disease did not occur in 64 uninoculated control birds kept under identical conditions. The largest number of chicks was injected with whole blood intraperitoneally: during two passages 46 per cent. of 52 birds developed the disease. It is recognised that the numbers are small, but the percentage of positive cases compares satisfactorily with that of Jungherr and Landauer (1938) who reported a take of 19·6 per cent. after inoculating 61 birds during four passages and with Brandley *et al.* (1942) who induced osteopetrosis in 9 per cent. of 884 birds inoculated from 1 to 22 days of age.

There is no indication as to why such a relatively large proportion of inoculated birds fail to develop the disease. It may be that some birds possess a high natural resistance. Brewer and Brownstein (1946), in discussing the incidence of lymphomatosis in different strains of fowls, concluded that one important factor may be the genetic susceptibility of the host to the disease. Other factors include the pathogenicity of the causative agent and the degree of exposure to it. As already mentioned, the field incidence is low, but often when cases do occur in a flock several birds are affected. Infection might appear in a flock in one or more of several ways; namely by egg transmission, through excreta from infected birds with clinical signs or through excreta from clinically normal birds which might act as carriers. The present experiments indicate the possibility that in the field the egg may act as one means of transmission of natural infection. Further studies are in progress on this aspect of the problem and will be reported later.

Brandley *et al* (1942), following transmission experiments, observed a significantly higher incidence in males and Burmester and Gentry (1955) from studies on their tumour strain RPL12 noted that males appeared to be slightly more susceptible to osteopetrosis. In this study there was a significantly higher incidence in male birds.

Three cases were not detected until post mortem at 6 months of age. It is interesting to note that in these birds, which had been inoculated with unfiltered plasma, the bone changes were cortical and medullary in type with negligible periosteal reactions, so that the conformation of the bones appeared normal on superficial examination and changes were only recognisable after radiography. This might explain the observations of Burmester and Gentry (1954) that clinical manifestation may remain latent in some passages and subsequently reappear, and emphasises the importance of radiological examination of all birds at death or at the end of the experimental period.

With one exception all the lesions were bilaterally symmetrical. In general they developed in exactly the same way as those observed in natural field outbreaks and became ultimately just as severe, with gross thickening of all the long bones. Following intra-amnion inoculation, lesions developed at the same age as in chicks inoculated at one day old.

## CONCLUSIONS

Experiments are described which demonstrate that avian osteopetrosis may be induced in normal fowls following the intraperitoneal injection into day old chicks of whole blood, bone marrow or unfiltered plasma from birds with the active disease.

The condition may also be induced by injection of blood into the amnion of the developing chick embryo.

These observations indicate that a transmissible agent is concerned in the etiology of osteopetrosis in the fowl and suggest that in field cases the egg may be one mode of transmission.

## ACKNOWLEDGMENTS

I wish to express my thanks to Miss A. Sparey for technical assistance throughout the experiment, Miss P. Pockson for help in rearing the chicks and Mr. M. Parsons for assistance with the radiography. The reproductions of the radiographs were very kindly prepared by Mr. J. E. Hancock to whom my sincere thanks are due. I am indebted to Dr. D. B. Peacock for help with the intra-amnion inoculations and Mr. J. Maffey who examined the dead-in-shell embryos. I also wish to thank Professor C. S. G. Grunsell and Dr. C. H. G. Price whose stimulating interest and encouragement in this work were invaluable.

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[*Received for publication, January 21st*, 1958]

Fig. 1

Radiographs from Donor No. 1. A.P. of tibia and metatarsal: (a) at 196 days; (b) at 238 days; (c) at 266 days; (d) at 308 days. Note the gradual increase in new periosteal bone, particularly in the metatarsal, and the extreme density of the tibia. This bird was used as a donor at 308 days and 319 days.

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Fig. 2

Fig. 3

Fig. 4

Fig. 2. Radiographs from Donor 91. A.P. of tibia and metatarsal: (a) at 49 days; (b) at 121 days. Note the very active periosteal proliferation particularly evident in the metatarsal. This bird was used as a donor at 51 days, at which time bone changes were not present in the metatarsals but were marked in the tibiae.

Fig. 3. Radiographs from Donor 64. A.P. of tibia and metatarsal: (a) at 50 days; (b) at 65 days; (c) at 146 days. Blood from this bird was used for transmission at 55 days. Note the initial changes on the tibia and later the typical periosteal proliferation of the metatarsal.

Fig. 4. Radiographs from Donor 68. A.P. of tibia and metatarsal: (a) at 50 days; (b) at 212 days. This bird was used as a donor at 55 days. Note the marked osteolysis in the metatarsal at 212 days. This was preceded by typical periosteal proliferation.

Fig. 5 Fig. 6

Fig. 5. Radiographs from Donor 65. (a) A.P. Tibia and metatarsal at 50 days; (b) lateral leg and wing at postmortem at 108 days. This bird was used as a donor at 55 days. This case was unusual in that the metatarsals never became involved.

Fig. 6. Radiograph from Donor 154. Tibia and metatarsal at 106 days. Used for plasma transmission at 132 days.

# THE PATHOLOGY OF EXPERIMENTAL STAPHYLOCOCCAL MASTITIS IN THE GOAT

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## INTRODUCTION

In a previous publication (Derbyshire, 1958) the clinical, bacteriological and haematological features of experimental staphylococcal mastitis in the goat were described. In this paper an account is given of the gross and histological appearance of the lesions in this experimental disease.

## MATERIALS AND METHODS

Goats were inoculated into the teat canal of one side of the udder only with living *Staphylococcus aureus* strain 201, or control substances, always contained in a volume of 10 ml. Details of the material examined are given in Table 1.

The histological method of examining these udders was that described by Pattison (1951), except that perfusion was not found to be a satisfactory method of fixing gangrenous udders. These were sliced immediately after slaughter, the slices immersed in 10 per cent formol-saline overnight, cut down into blocks about 15 mm. square and 5 mm. thick on the following day, and fixation continued for several days. One section from all blocks was stained by haematoxylin and eosin, and one by Gram's method.

## RESULTS

### *Lesions Produced by Non-living Inocula*

The lesions produced by the control substances listed in Table 1 were confined to a mild histological abnormality detected only in the goats slaughtered two days after inoculation. There was a slight patchy migration of neutrophils into occasional secretory alveoli and parts of the walls of the large ducts showed a mild degree of subepithelial infiltration with neutrophils and lymphocytes.

### *Lesions in Gangrenous Mastitis*

*Gross changes.* The inoculated side of the udder was enlarged and swollen, and showed a purplish to black discolouration of the skin. The subcutaneous connective tissue and the interlobular septa were grossly oedematous, and the subcutaneous oedema extended anteriorly along the ventral surface of the abdomen on the side corresponding with the inoculated side of the udder, but never extended across the linea alba. The galactophorous sinus of the inoculated side of the udder contained blood-stained fluid. The non-inoculated gland was macroscopically normal.

*Histopathology.* In the goat killed six hours after inoculation, massive numbers of staphylococci (Fig. 1) had penetrated to alveoli widely distributed in the mammary tissue and there was a widespread migration of neutrophils from dilated capillary blood vessels into the alveoli (Fig. 2). There were degenerative changes in the epithelial cells of the alveoli and small ducts, varying from vacuolation to necrosis and sloughing of the epithelium. From 24 hours after inoculation, at the stage when the disease was recognisable clinically as "gangrenous" mastitis, evidence of thrombus formation could be found in the interlobular veins, and in the main veins at the dorsal aspect of the inoculated half of the udder. Early thrombi consisted of interlacing strands of platelets and fibrin (Fig. 3) and later the bands of platelets were coated with polymorphs. The endothelial lining of these blood vessels was eroded, and there was a sub-endothelial infiltration of polymorphs and small mononuclear cells (Fig. 4). Cocci were not found in direct association with thrombosed veins.

TABLE 1

DETAILS OF MATERIAL EXAMINED

| *Inoculum* | *Time between inoculation and slaughter* | *No. of goats* | *No. of blocks examined* | |
|---|---|---|---|---|
| | | | *Inoculated side* | *Non-inoculated side* |
| Uninoculated broth .. .. | 2 and 9 days | 2 | 47 | 8 |
| Heat-killed staphylococci .. .. | 2 and 9 days | 4 | 79 | 12 |
| Chamberland L2 filtrate of culture | 2 and 9 days | 2 | 32 | 6 |
| Living staphylococci, producing gangrenous mastitis .. | 6 hours | 1 | 19 | 3 |
| " " " .. | 1 day | 1 | 25 | 3 |
| " " " .. | 2 days | 2 | 48 | 6 |
| " " " .. | 3 days | 1 | 8 | 3 |
| " " " .. | 6 days | 1 | 10 | 4 |
| " " " .. | 8 days | 1 | 14 | 3 |
| Living staphylococci, producing non-gangrenous mastitis | 3 days | 1 | 29 | 3 |
| " " " | 8 days | 1 | 19 | 3 |
| " " " | 9 days | 1 | 19 | 3 |
| " " " | 15 days | 1 | 19 | 3 |
| " " " | 30 days | 1 | 30 | 4 |
| " " " | 78 days | 1 | 26 | 3 |
| | Total | 21 | 424 | 67 |

Once thrombo-phlebitis had developed, inflammatory changes in the mammary tissue were arrested, but degeneration continued so that the alveolar structure of the lobules was lost (Fig. 5). Sloughed, necrotic epithelial cells with large numbers of staphylococci were

present in the small ducts and within folds of the large ducts (Fig. 6). The epithelial lining of the large ducts also sloughed, but the epithelial polymorph infiltration was slight and patchy. In a late stage of the disease widespread areas of the inoculated gland were completely necrotic.

In the non-inoculated side of the udder changes were limited to a widespread stasis of milk secretion.

*Lesions in Non-gangrenous Mastitis*

In goats in which gangrenous changes did not occur, the degree of reaction to the inoculation of living staphylococci varied from mild to severe. No abnormality occurred in the non-inoculated side of the udder in these goats.

*Mild Reaction*

At post-mortem examination, no gross differences between the inoculated and non-inoculated sides of the udder were detected. Histologically, the changes were indistinguishable in character from the non-specific reaction which occurred when control substances were introduced into the teat canal, but they tended to be rather more widespread. Very small numbers of staphylococci were occasionally found in folds of the large ducts, but never in the small ducts or in the alveoli.

*Severe Reaction*

Prominent macroscopic lesions occurred in the inoculated glands. These were focal abscesses up to 3 cm. in diameter, consisting of a creamy necrotic centre surrounded by annular fibrous tissue. The abscesses were distributed irregularly in the substance of the mammary gland and did not appear to be related to the duct system. Histological changes are described below.

*Changes in the lobules.* The earliest change to be detected was an extensive migration of neutrophils from the blood vessels into the alveoli although, in contrast to gangrenous mastitis, this involved only parts of the udder so that a variable proportion of normal, functional tissue always remained. Within three days of inoculation, degenerative changes in the alveoli, in the form of intense epithelial cell vacuolation, were observed and concurrently there was an interstitial infiltration with macrophages and fibroblasts, which in places tended to obliterate the alveolar structure of the lobules. Eight days after inoculation, focal and diffuse infiltrations of lymphocytes were numerous in the inter-alveolar tissue (Fig. 7). Later, involutionary changes accompanied the increasing fibrosis (Fig. 8).

Abscess formation was the result of more severe changes in the lobules. It appeared to be initiated by the breakdown of a centri-lobular duct containing massive numbers of staphylococci, followed by necrosis of the alveoli of the lobule, and often extended to surrounding lobules. Such areas were walled-off by a dense

infiltration of polymorphs in the interlobular septa, but the lesions usually extended to involve considerable areas of mammary tissue. Later, the polymorph zone was circumscribed by dense fibrous tissue, and at this stage the centre of the lesion consisted of necrotic alveoli and degenerating polymorphs, (Fig. 9), with massive clumps of staphylococci. This type of lesion harbouring large numbers of organisms was still present in the goat killed 78 days after inoculation.

*Changes in the ducts*. The smaller ducts were dilated and sometimes contained ingrowths of granulation tissue (Fig. 10) covered with epithelium which was often stratified. The lumen of such a duct invariably contained small numbers of polymorphs and cocci. The ingrowths increased in size between the eighth and thirtieth days after inoculation, but were later shed. These lesions were identical with those described by Pattison (1951) in streptococcal mastitis in the goat. The epithelial lining of the large ducts showed patches of hyperplasia at an early stage and later became stratified (Fig. 11). Small numbers of cocci and polymorphs could usually be found in folds of these ducts. There was a corresponding sub-epithelial infiltration of the duct wall with polymorphs, lymphocytes and fibroblasts. In the late stage of the disease the stratified epithelium was lost, but well-developed foci of lymphocytes occurred in the duct walls (Fig. 12).

## DISCUSSION

The literature on the pathology of bovine staphylococcal mastitis has been reviewed by Helmboldt, Jungherr and Plastridge (1953). It is clear that the experimental disease in the goat resembles the natural disease in cattle very closely. Pattison (1951) has described the histological findings in experimental streptococcal mastitis in the goat and these resemble the non-gangrenous mastitis described here, except that streptococci did not cause abscess formation. Gangrenous mastitis was not produced by streptococci.

The occurrence of thrombi in the veins of the udder in bovine necrotic-gangrenous mastitis was reported by Nieberle and Cohrs (1931). Renk (1957) has described venous thrombosis in acute coliform mastitis in the cow and goat. The results of the present investigation suggest that thrombosis of the main veins draining the infected mammary gland is of fundamental significance in the pathogenesis of gangrenous mastitis caused by staphylococci. The thrombus formation is associated with a rapid multiplication of staphylococci in the adjacent mammary tissue with the liberation of toxic substances, so that the lesion is comparable with thrombosis of tributaries of the mesenteric vein in acute appendicitis in man (Payling-Wright, 1950). In these goats, occlusion of the mammary veins is probably the cause of the oedema of the ventral abdominal wall, as well as the mammary gland oedema, due to deficient drainage by the posterior subcutaneous abdominal vein on the affected side.

The observations on these experimental goats have some clinical

significance, since they have emphasized the great speed with which irreversible tissue changes occur—within 24 hours of inoculation in gangrenous mastitis. Also, in non-gangrenous mastitis, living staphylococci may be rapidly surrounded by dense fibrous tissue in such a position as to be inaccessible to drugs injected into the teat canal. Thus, even though the infecting strain of staphylococcus may be susceptible to the drug used, the latter may fail to reach infective foci within the udder.

## CONCLUSIONS

Udder sections were examined from thirteen goats inoculated with living staphylococci into the teat canal and from eight goats similarly inoculated with control substances and slaughtered at intervals after inoculation. The control substances produced a transient non-specific reaction only and this was similar to the histological changes observed in mild, non-gangrenous mastitis produced by living staphylococci.

In gangrenous mastitis there was widespread, rapid necrosis of the mammary tissue associated with thrombosis of the veins draining the inoculated side of the udder. In severe, non-gangrenous mastitis lobular changes consisted of degeneration of the epithelial cells of many alveoli and an interstitial inflammatory reaction leading to extensive fibrosis with involution of the secretory alveoli. Abscess formation frequently occurred. Large ducts showed epithelial stratification and ingrowths of granulation tissue occurred in smaller ducts.

The pathology of this experimental staphylococcal mastitis resembled the natural bovine disease as reported in the literature.

## ACKNOWLEDGMENTS

I wish to thank Mr. I. H. Pattison for his constant interest and advice in this work. It is a pleasure to acknowledge the technical assistance of Mr. F. H. Summerfield, who prepared the photomicrographs, and of Mr. P. F. Dennis and Miss J. L. Dowle.

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[*Received for publication, January 22nd,* 1958]

## LEGENDS TO FIGS.

Fig. 1. Clumps of cocci in alveoli (6 hours after inoculation) Gram. × about 320.

Fig. 2. Migration of neutrophils into alveoli (6 hours after inoculation). H. and E. × about 200.

Fig. 3. Early thrombus in a vein (2 days after inoculation). H. and E. × about 100.

Fig. 4. Venous thrombosis, platelet strands coated with polymorphs, and sub-endothelial mononuclear infiltration (2 days after inoculation). H. and E. × about 75.

Fig. 5. Degeneration of mammary tissue; massive numbers of cocci. (2 days after inoculation). Gram. × about 200.

Fig. 6. Large duct; loss of epithelium; necrotic cells and cocci in lumen. (2 days after inoculation) H. and E. × about 200.

Fig. 7. Inter-alveolar infiltration with lymphocytes. (8 days after inoculation) H. and E. × 320.

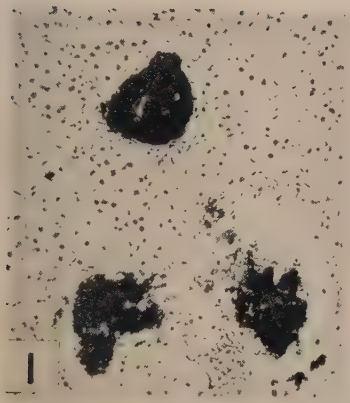
Fig. 8. Fibrosis and involution (30 days after inoculation). H. and E. × about 200.

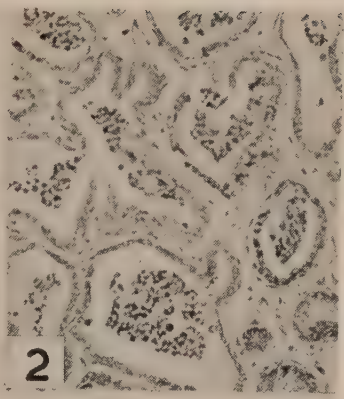
Fig. 9. Necrotic centre of an abscess (15 days after inoculation). H. and E. × about 150.

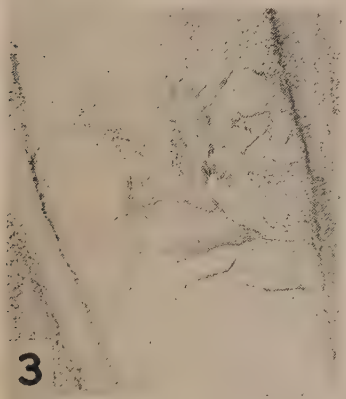
Fig. 10. Ingrowth of granulation tissue in a small duct (15 days after inoculation). H. and E. × about 150.

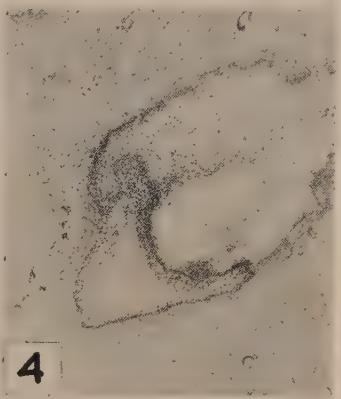
Fig. 11. Epithelial stratification in a large duct (9 days after inoculation). × about 200.

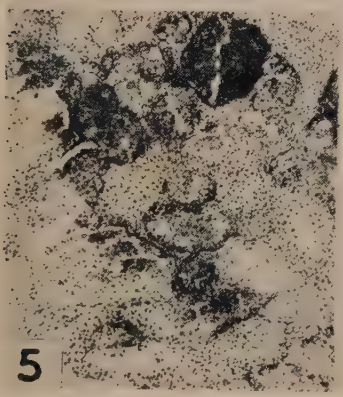
Fig. 12. Lymphocyte focus in wall of large duct (78 days after inoculation). H. and E. × about 200.

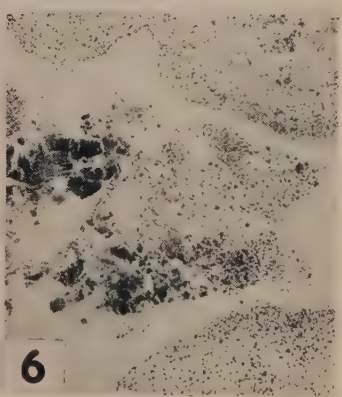
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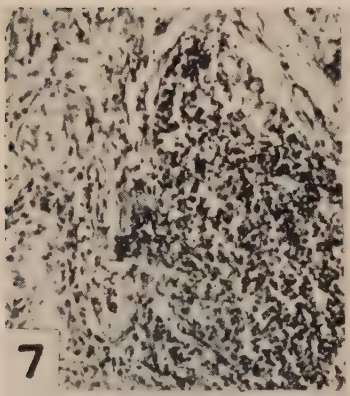
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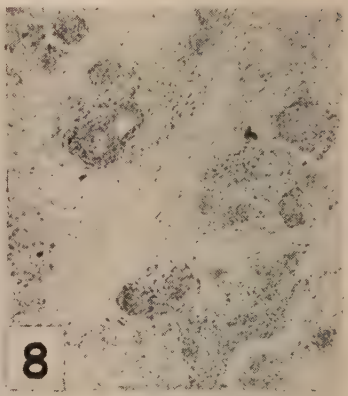
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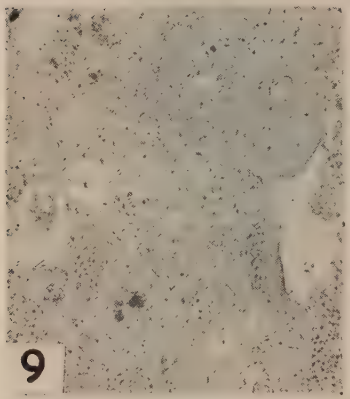
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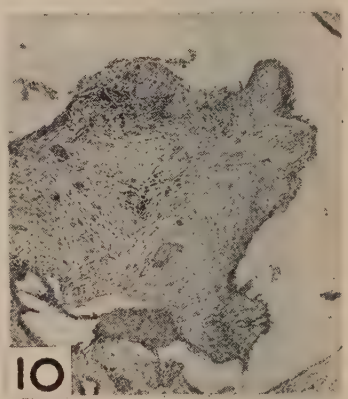
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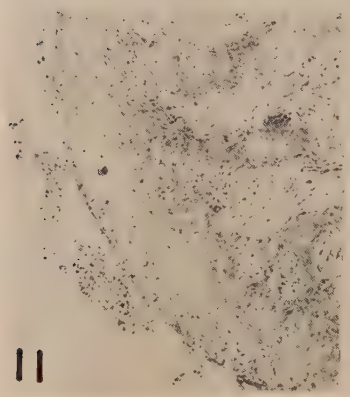
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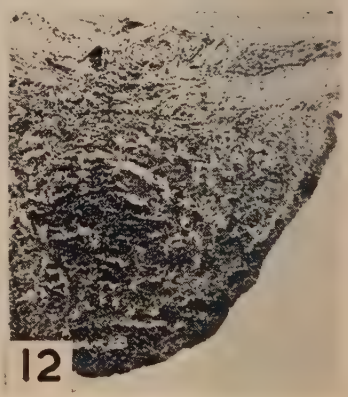
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# EXPERIMENTAL INFECTIONS OF *PASTEURELLA HAEMOLYTICA* IN MICE AND THEIR USE IN DEMONSTRATING PASSIVE IMMUNITY

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## INTRODUCTION

*Pasteurella haemolytica*, so named by Newsom and Cross (1932), and organisms isolated from sheep and cattle which correspond more or less to the description given by these authors, have long been known to possess little pathogenicity for laboratory animals when administered by conventional methods.

The Group I organisms of Jones (1921) produced only a slight local reaction in rabbits when injected subcutaneously in a dose of 0·25 ml. of 24 hour bouillon culture, and 0·1 ml. given by the same route failed to kill mice. After a few generations on artificial media 0·5 ml. injected intraperitoneally into mice was also without effect. During the course of many attempts to infect rabbits by the intravenous, intraperitoneal and subcutaneous routes, Tweed and Edington (1930) succeeded in producing obvious disease and death from septicaemia in only one animal, and this by means of a large dose of about 10,000 x $10^6$ freshly isolated bacteria. Dungal (1931) obtained a similar though non-haemolytic organism from diseased sheep, and although it killed two mice within 20 hours of the intraperitoneal injection of 0·5 ml. of an 18 hour broth culture, rabbits survived intravenous, intratracheal and subcutaneous injections of 1 ml. of 12 hour bouillon culture, and guinea-pigs survived 1 ml. given subcutaneously. Cats, dogs, hens and pigeons were not affected. Lovell and Hughes (1935) in a study of five strains found that the intraperitoneal injection of 1 ml. of a 24 hour broth culture failed to kill guinea-pigs, while 0·5 ml. by the same route caused death in mice in 24 to 48 hours, and they concluded that the virulence for laboratory animals was low. Beveridge (1937) in studying an outbreak of disease in sheep in Australia reported that the organism concerned was without lethal effect in mice in subcutaneous doses of up to 0·5 ml. of serum broth culture, while rabbits and guinea-pigs also resisted large doses. Montgomerie, Bosworth and Glover (1938) found that rabbits tolerated doses of up to 2 ml. of 24 hour broth culture by intravenous, subcutaneous or intraperitoneal routes. Guinea-pigs and pigeons survived large doses given by a variety of routes, while mice succumbed in 24 hours to 3 days to comparatively large doses (0·3 to 0·5 ml. of 24 hour broth culture) given intraperitoneally, but these strains soon lost their pathogenicity on subcultivation. The organism was without effect by other routes, and the minimum lethal dose in mice was not appreciably altered by rapid serial intraperitoneal injections. The work of Rosenbusch and Merchant (1939) and of Francis (1941) adds further support to the view that these organisms are of low virulence for mice. Stamp, Watt and Thomlinson (1955) reported the reproduction of a septicaemic disease in lambs by the intravenous injection of tissue suspensions and cultures of *Past. haemolytica*, but they pointed out that the high

cost of the sheep as an experimental animal precluded detailed investigation of pathogenicity.

Thus, in the past, the apparent lack of a suitable experimental animal has severely curtailed studies of *Past. haemolytica*. The purpose of this paper is to record progress in the investigation of two methods by means of which true infections can be established in mice, with doses considerably smaller than hitherto, characterized by marked multiplication of the organism and affording a means of demonstrating protection by immune sera. These two methods are (a) intraperitoneal injection using granular mucin as an aid to infection, and (b) intracerebral injection.

## MATERIALS AND METHODS

*Strains of* Past. haemolytica. All strains used in the following experiments were isolated from sheep in Scotland with the exception of No. 32 which was obtained from the pneumonic lung of a lamb in S. Wales. Nos. 8 and 36 were isolated from cultures of nasal swabs taken from apparently healthy sheep (No. 36 was the predominating organism from the swab). No. 34 was from a severe case of pneumonia in an adult sheep, and Nos. 1118 and 7157H were cultured from the blood and organs of two lambs which died from a septicaemic condition. These six strains were the only ones examined in any detail, but from more empirical observations on a considerable number of other strains they appeared to be typical as regards their mouse pathogenicity. No. 7157H was the only strain used for protection experiments and it was selected at random for this purpose. A culture made from the heart blood of an experimentally infected mouse was freeze-dried for use in the intraperitoneal protection tests. For challenge purposes in intracerebral protection experiments No. 7157H was freeze-dried after the forty-second consecutive intracranial mouse passage.

*Bacterial suspensions for inoculation.* Unless stated otherwise, all inocula consisted of 18 to 20 hour growths on 5 per cent sheep blood agar, scraped off and evenly suspended in a solution of 1 per cent casein hydrolysate in distilled water, pH 7·1. These suspensions were then suitably diluted in casein hydrolysate solution and in the case of intraperitoneal experiments mixed with mucin. The temperature in all instances was maintained at 4°C until shortly before the time of injection. Wherever it was necessary to inject mice with a reasonably accurate dose of bacteria, advantage was taken of the fairly constant low death rate of *Past. haemolytica* in 1 per cent casein hydrolysate solution at 4°C. It was found that, in general, a loss in viability of about 25 per cent could be expected when suspensions were maintained under these conditions for 24 hours. Accordingly, a suspension could be prepared on Day 1 and a viable count made. The result of the count would be available on Day 2 (the day of inoculation) and the suspension could then be diluted to contain the required number of living bacteria per millilitre, allowing for a 25 per cent loss in viability. The results of a further viable count on this adjusted suspension performed just before inoculation would then be available on Day 3 as a retrospective check. In practice, this method was quite satisfactory and was used in all the following experiments with the exception of the intracerebral virulence

titrations, in which organisms were harvested from blood agar and inoculated on the same day.

*Viable counts.* Ten-fold dilutions of suspensions were made up in casein hydrolysate solution by transferring 0·1 ml. of suspension into 0·9 ml. of diluent, using a separate 1 ml. pipette for each dilution. A dropping pipette calibrated to deliver 0·02 ml. per drop (Miles and Misra, 1938) was used to sample each dilution, the drop being placed on the surface of sectored blood agar plates. Colonies were counted after 18 hours incubation. Five replicate counts were performed in each case and the average taken as the true count.

*Sera.* Hyperimmune serum against strain No. 71571H was prepared in two rabbits, each of which received a course of intravenous injections of formalin-killed bacteria, followed by increasing doses of living organisms. A proportion of these injections consisted of organisms which had been passaged intracerebrally a number of times in mice. The maximum immunising dose used was 0·45 ml. of living 24 hour broth culture. Agglutinin titres were followed in these rabbits using a stock formalinized suspension of strain No. 71571H, diluted to Brown's tube 3·5 opacity before use. Agglutination tests were incubated at 55°C for 2 hours and all protection experiments were performed after the two sera had reached their maximum titre, which in both cases was 1/1024. Normal rabbit sera, checked for absence of agglutinins, were used for control mice. All sera were sterilized by filtration and were used two days after collection from the rabbits. None was inactivated by heat. Serum dilutions were made up in normal saline and were injected intraperitoneally, in a dose volume of 0·5 ml., 5 to 6 hours before challenge.

*Mice.* Young Swiss white mice from the Moredun Institute closed colony were used. Those for intracerebral inoculation weighed from 15 to 20 gm., while those used in intraperitoneal experiments were somewhat larger, ranging from 17 to 22 gm. The sexes were evenly distributed.

*Intracerebral inoculations.* Injections of organisms suspended in casein hydrolysate solution were made under ether anaesthesia. Doses of 0·05 ml. were delivered from a 0·25 ml. syringe with a 26-gauge needle.

*Intraperitoneal inoculations incorporating mucin.* Granular mucin, type 1701–W*, was made up at the rate of 5 gm. of mucin per 100 ml. of distilled water, and was prepared, autoclaved and neutralized according to the manufacturer's instructions. This mucin suspension was used in the proportion of 4 parts of mucin to 1 part of bacterial suspension. The dose volume per mouse of this inoculum was standardized at 0·5 ml. Provided that organisms were omitted, such an inoculum was shown to be well tolerated by mice. Dilution series of organisms for intraperitoneal inoculations were never prepared by using mucin suspension as the diluent for fear of errors, due to mixing in a rather viscous fluid, being transmitted down the series. Instead, dilution series were prepared in casein hydrolysate solution, a measured volume from each dilution tube being transferred to 4 times this volume of mucin suspension and mixed very thoroughly with a pipette. Where parallel control mice were to be inoculated with organisms only and not mucin, as in the experiments designed to demonstrate the capacity of mucin for reducing the resistance of mice to *Past. haemolytica*, a measured volume from each dilution tube was

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transferred into 4 times this volume of casein hydrolysate solution. The dose volume of such control inocula was again 0·5 ml.

## RESULTS

### *Intraperitoneal Infection using Granular Mucin*

Preliminary observations on mice confirmed the conclusions of earlier workers as to the low virulence of *Past. haemolytica* when administered by conventional methods. An interesting point which emerged from these initial investigations was the discovery that mice are capable of withstanding very much larger doses of *Past. haemolytica* intravenously than intraperitoneally. Mucin preparations have been used in the past for reducing the resistance of experimental animals to a number of micro-organisms whose virulence is such that inordinately large numbers of bacteria are necessary to produce lethal effects. Much of this work has been reviewed by Olitzki (1948).

The present study has shown that the numbers of *Past. haemolytica* required to produce fatal infections in mice by intraperitoneal injection are markedly reduced if mucin is incorporated in the inoculum. A rapidly fatal septicaemia results, the great majority of deaths occurring within 48 hours of inoculation. Smears of peritoneal fluid and heart blood of dead mice generally show very large numbers of bacteria per field on microscopic examination. Usually the organisms are quite regular in shape and size, but smears from a particular mouse occasionally show very marked pleomorphism, which may take the form of grossly thickened or elongated bacteria or globular forms similar to those of *H. influenzae* described by Pittman (1931).

Table 1 shows the results of titrations of 5 strains over a wide range of decimal dilutions, with and without the use of mucin. Cultures were made from each dead mouse to confirm that it had died from a pure infection of *Past. haemolytica*. The largest inoculum of each strain was made to contain approximately 1000 x $10^6$ viable organisms in order that rough comparisons between strains might be possible. It will be seen that mucin lowered the resistance of mice to all strains tested, but to a greater degree for some than others. The very large dose necessary to produce deaths in mice not receiving mucin is also demonstrated.

That multiplication of the organism occurs when it is injected intraperitoneally together with mucin was conclusively demonstrated by the rapid serial passage of strain No. 7157H direct from mouse peritoneum to peritoneum. Peritoneal washings in casein hydrolysate solution were collected from dead mice, and a small portion of each washing was injected intraperitoneally together with mucin into each of two mice, one of which received a rather larger inoculum than the other. In this way it was always possible in practice to post-mortem at least one animal shortly after death, thus avoiding contamination. Inocula were regularly checked for purity by aerobic

and anaerobic culture. Twenty-four consecutive mouse passages were obtained without difficulty. After these 24 passages it was no longer necessary to incorporate mucin in the inoculum and a further 24 passages were achieved by injecting small portions of peritoneal washings alone, after which the experiment was discontinued. It appeared that for this adaptation to the mouse peritoneum the use of mucin in the early passages was essential. Three subsequent attempts to so adapt the original strain 7157H without the use of mucin failed. The initial inocula were massive doses of broth culture and large amounts of each peritoneal washing were used as inocula. However, the best that was achieved in this way was 5 consecutive passages before the organism died out.

TABLE 1

INTRAPERITONEAL TITRATIONS

5 strains of *Past. haemolytica* in mice, with and without the use of Mucin

| | | *Deaths in groups of 4 mice using dilutions of organisms shown below* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Strain No.* | *Mucin* | *Maximum dose* | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ |
| 32 | — | 1 | 0 | 1 | 1 | N | N | N | N | N |
| | + | N | 4 | 4 | 2 | 1 | 1 | 0 | 0 | 0 |
| 34 | — | 1 | 0 | 0 | 0 | N | N | N | N | N |
| | + | N | 4 | 3 | 2 | 3 | 1 | 0 | 0 | 0 |
| 36 | — | 2 | 2 | 1 | 0 | N | N | N | N | N |
| | + | N | 4 | 3 | 4 | 4 | 3 | 2 | 0 | 0 |
| 1118 | — | 2 | 1 | 0 | 0 | N | N | N | N | N |
| | + | N | 4 | 2 | 3 | 2 | 1 | 1 | 1 | 0 |
| 7157H | — | 4 | 1 | 0 | 0 | N | N | N | N | N |
| | + | N | 4 | 2 | 2 | 0 | 0 | 0 | 0 | 0 |

N = not done.

Viable counts showed maximum doses to contain the following numbers of organisms

Strain 32 $1030 \times 10^6$
,, 34 $1125 \times 10^6$
,, 36 $1090 \times 10^6$
,, 1118 $1030 \times 10^6$
,, 7157H $1025 \times 10^6$

*Intraperitoneal Protection Tests using Mucin*

The objects of this part of the work were to show (1) that a hyperimmune rabbit serum could be prepared which would protect mice against intraperitoneal challenge with *Past. haemolytica* in mucin; (2) that such a serum could be titrated in mice against a constant challenge dose, and (3) the effect of using graded challenge doses against a single dose of serum.

Preliminary experiments indicated the approximate range of

challenge dose of strain No 7157H over which immune antibody effects might be demonstrated. This work was repeated subsequently by a somewhat modified experiment, the results of which are recorded later in this paper. Initially, however, it was desirable to gain at least a rough knowledge of that dose of immune serum which would be large enough to produce a high level of immune antibody in mice consistent with the elimination of any undesirable attributes of rabbit serum which might become manifest if too large a dose were used.

TABLE 2

THE EFFECTS OF LARGE DOSES OF HYPERIMMUNE AND NORMAL RABBIT SERA

Mice challenged intraperitoneally with *Past. haemolytica* (Strain 7157H)

| | *Serum dilutions* | *Hours after challenge* | *Deaths in groups of 20 mice using 2 challenge levels* | | *Total deaths* |
|---|---|---|---|---|---|
| | | | *75 × 10⁶ organisms* | *37·5 × 10⁶ organisms* | |
| Immune serum | 1/1 | 24 | 2 | 0 | 2/40 |
| | | 72 | 8 | 8 | 16/40 |
| | 1/4 | 72 | 2 | 1 | 3/40 |
| | 1/14 | 72 | 4 | 1 | 5/40 |
| | 1/100 | 24 | 15 | 7 | 22/40 |
| | | 72 | 16 | 7 | 23/40 |
| Normal serum | 1/1 | 24 | 3 | 1 | 4/40 |
| | | 72 | 10 | 6 | 16/40 |

Dose volume of serum dilutions = 0·5 ml. intraperitoneally.
Challenge inocula incorporated mucin.

Information on these points was supplied by an experiment the results of which are shown in Table 2. Groups of mice received varying doses of hyperimmune and normal rabbit serum prior to challenge with organisms in mucin. The experiment was duplicated using a second challenge dose half the size of the first. This reduced the risk of supplying an unsuitable dose of organisms due to technical errors, a risk which might be very real if only a single dose level were used. Thus one could choose to interpret the test either on the basis of deaths in mice at single challenge levels or on the basis of deaths at both levels combined, whichever might be more advantageous. The practical benefits of this procedure are, in fact, better demonstrated in the next test to be described. Deaths were recorded 24 hours after challenge and again at 72 hours, after which time no further deaths occurred. The results show that 0·5 ml. of undiluted normal rabbit serum is capable of providing protection to mice as compared with 0·5 ml. of 1/100 immune rabbit serum, but under

the conditions of the experiment this effect is manifested as a prolongation of survival time and tends to be obliterated as the test progresses towards completion. At the same time it is apparent from a comparison of final deaths of mice receiving 1/1 and 1/4 immune serum that 0·5 ml. of undiluted rabbit serum also exerts an eventual lethal effect. This effect is probably better thought of as decreasing the resistance of mice to the experimental infection since 10 mice which were subsequently given a similar intraperitoneal dose of rabbit serum alone merely showed a slight loss in weight from which they rapidly recovered. It would seem from these results that in choosing a single dose of hyperimmune rabbit serum for the purpose of demonstrating protection in mice under optimal conditions it would be unwise to exceed 0·5 ml. of a 1/4 dilution, and, indeed, quite unnecessary since a 1/14 dilution is also adequate.

Table 3 shows the results of titration of hyperimmune rabbit serum in mice. The method used was similar to that of the previous experiment except that the groups of mice were smaller. A control titration of normal rabbit serum was performed in parallel and under identical conditions. Mice receiving 0·5 ml. of saline instead of serum prior to challenge were also included. The upper challenge level of 90 x $10^6$ viable organisms in mucin was capable of demonstrating protection by immune serum only as far as the 1/14 dilution. The smaller challenge dose however was more successful, and combining the deaths obtained using it against both the 1/100 and 1/200 dilutions of sera it is clear that protection was still present at the 1/100 dilution of immune serum.

TABLE 3

COMPARATIVE TITRATIONS OF HYPERIMMUNE AND NORMAL RABBIT SERA IN MICE

Intraperitoneal challenge with *Past. haemolytica* (Strain 7157H)

| *Serum dilutions* | | 1/4 | | 1/14 | | 1/50 | | 1/100 | | 1/200 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Type of serum* | | *I* | *N* | *I* | *N* | *I* | *N* | *I* | *N* | *I* | *N* | *Saline* |
| Deaths in groups of 10 mice using 2 challenge levels | 90 × $10^6$ organisms | 2 | 9 | 0 | 8 | 7 | 10 | 9 | 9 | 9 | 8 | 9 |
| | 45 × $10^6$ organisms | 0 | 5 | 1 | 8 | 1 | 10 | 5 | 10 | 5 | 10 | 10 |
| Total Deaths | | 2/20 | 14/20 | 1/20 | 16/20 | 8/20 | 20/20 | 14/20 | 19/20 | 14/20 | 18/20 | 19/20 |

I = Hyperimmune rabbit serum.
N = Normal rabbit serum.
Dose volume of serum dilutions = 0·5 ml. intraperitoneally.
Challenge inocula incorporated mucin.

The results of the final experiment in this section are recorded in Table 4. The purpose in this case was to study the effect of using

graded challenge doses of organisms in mucin against a constant dose of serum. Each challenge level was tested against normal and hyperimmune rabbit serum. Serum was injected intraperitoneally in volumes of 0·5 ml. of a 1/10 dilution, this dose being chosen on the basis of information provided by experiments already described. Organisms were injected in two-fold dilutions since effective challenge doses were known to be high. The immune serum gave a very high degree of protection over a particular range of challenge, the order of which is indicated by the Table.

TABLE 4

1/10 DILUTIONS OF HYPERIMMUNE AND NORMAL RABBIT SERA

Graded intraperitoneal challenge doses of *Past. haemolytica* (Strain 7157H) in mice

| | *Deaths in groups of 12 mice using challenge doses shown below* | | | | |
|---|---|---|---|---|---|
| | $140 \times 10^6$ | $70 \times 10^6$ | $35 \times 10^6$ | $17{\cdot}5 \times 10^6$ | $8{\cdot}75 \times 10^6$ |
| 1/10 immune serum | 4 | 0 | 1 | 0 | 0 |
| 1/10 normal serum | 12 | 11 | 10 | 1 | 0 |

Dose volume of 1/10 serum = 0·5 ml. intraperitoneally.
Challenge inocula incorporated mucin.

*Intracerebral Infection*

All strains of *Past haemolytica* tested have proved capable of infecting mice by the intracerebral route. Experiments have shown that mice destined to die almost always do so within 10 days, although very occasionally deaths occur up to the 14th day. The symptoms are typical of a cerebral infection and at death the organisms are usually confined to the brain and meninges, although occasionally a loopful of heart blood will give rise to one or two colonies when cultured on blood agar. Smears from brain tissue generally reveal very large numbers of bacteria on microscopic examination. Pleomorphism is usually either absent or slight. Counts of viable organisms in brain tissue suspensions, prepared by disintegrating whole weighed brains in measured volumes of 1 per cent. casein hydrolysate solution in Griffith tubes, reveal that multiplication occurs to a high degree. For example, the brain of one mouse which died on the 6th day after receiving an estimated dose of 230 organisms contained $12{\cdot}2 \times 10^6$ viable bacteria at death. The brain of another mouse contained $2000 \times 10^6$ viable organisms at death which occurred within 24 hours of the injection of $2{\cdot}75 \times 10^6$ organisms.

Table 5 shows the results of virulence titrations of five different

strains. With the exception of No. 7157H which was tested immediately after the 34th consecutive intracerebral mouse passage, these strains had been maintained in artificial culture for varying periods in the laboratory. It will be seen that the susceptibility of mice varies considerably, some dying after a dose of less than 100 viable organisms, others resisting a dose 10,000 times as great. With the numbers of mice used even 100-fold dose differences frequently resulted in little or no change in mortality. The $LD_{50}$ would in general appear to be in the low thousands.

TABLE 5

INTRACEREBRAL VIRULENCE TITRATIONS OF 5 STRAINS OF *PAST. HAEMOLYTICA* IN MICE

| *Strain No.* | *Dose of viable organisms* | *Showing the days after inoculation on which each individual mouse died* | | | | | | | | | | | | *Total deaths* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 | $10 \times 10^5$ | 1 | 1 | 1 | 1 | 2 | 3 | | | | | | | 6/6 |
| | $10 \times 10^4$ | 2 | 3 | 4 | 4 | 4 | 4 | 5 | 5 | 6 | 7 | S | S | 10/12 |
| | $10 \times 10^3$ | 2 | 3 | S | S | S | S | S | S | S | S | S | S | 2/12 |
| | 10 | 2 | 2 | S | S | S | S | S | S | S | S | S | S | 2/12 |
| | 0 | S | S | S | S | S | S | | | | | | | 0/6 |
| 32 | $46 \times 10^6$ | 1 | 1 | 1 | 1 | 2 | 4 | | | | | | | 6/6 |
| | $46 \times 10^4$ | 3 | 4 | 5 | 5 | 5 | 6 | 7 | S | S | S | S | S | 7/12 |
| | $46 \times 10^2$ | 1 | 3 | 3 | 5 | 5 | S | S | S | S | S | S | S | 5/12 |
| | 46 | 4 | 4 | 4 | 4 | X | S | S | S | S | S | S | S | 4/11 |
| | 0 | S | S | S | S | S | S | | | | | | | 0/6 |
| 34 | $17 \times 10^6$ | 1 | 1 | 1 | 2 | 2 | 2 | | | | | | | 6/6 |
| | $17 \times 10^4$ | 1 | 1 | 2 | 4 | 4 | 4 | 5 | 5 | 6 | 6 | S | S | 10/12 |
| | $17 \times 10^2$ | 2 | 3 | 3 | 3 | 4 | S | S | S | S | S | S | S | 5/12 |
| | 17 | 6 | S | S | S | S | S | S | S | S | S | S | S | 1/12 |
| | 0 | S | S | S | S | S | S | | | | | | | 0/6 |
| 36 | $83 \times 10^6$ | 1 | 2 | 2 | 2 | 5 | 6 | | | | | | | 6/6 |
| | $83 \times 10^4$ | 1 | 2 | 4 | 4 | 6 | 6 | 7 | S | S | S | S | S | 7/12 |
| | $83 \times 10^2$ | 1 | 1 | 1 | 2 | 5 | 5 | 6 | S | S | S | S | S | 7/12 |
| | 83 | 1 | 2 | S | S | S | S | S | S | S | S | S | S | 2/12 |
| | 0 | S | S | S | S | S | S | | | | | | | 0/6 |
| 7157H (Passaged) | $57 \times 10^6$ | 1 | 1 | 1 | 1 | 1 | 2 | | | | | | | 6/6 |
| | $57 \times 10^4$ | 1 | 2 | 3 | 3 | 3 | 4 | 5 | 6 | 6 | S | S | S | 9/12 |
| | $57 \times 10^2$ | 1 | 1 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | S | S | S | 9/12 |
| | 57 | 3 | 4 | 4 | 5 | 7 | S | S | S | S | S | S | S | 5/12 |
| | 0 | S | S | S | S | S | S | | | | | | | 0/6 |

S = Mouse survived.
X = Mouse died but brain sterile.

*Modified method of producing cerebral infections.* This modification is similar to that used by Sawyer and Lloyd (1931) in their work on yellow fever.

The present investigations have shown that a sub-lethal intravenous dose of *Past. haemolytica* administered to a mouse can be con-

verted into a lethal dose by subsequent brain trauma. Injury to the brain can be effected by a number of treatments, one of which consists of the intracerebral injection of 0·03 ml. of 2 per cent. starch-saline solution. Such a treatment is, in itself, well tolerated by mice. Mice die within 10 days showing symptoms of a cerebral infection and at post-mortem smears of brain tissue show very large numbers of bacteria. Frequently culture indicates that organisms are confined to the brain, but in other cases considerably smaller numbers may be cultured from heart blood and spleen also.

It appears to be preferable to use a strain which has been passaged intracerebrally a number of times in mice for this method of infection. Using such a strain in a suitable dose it is possible to obtain a mortality of 100 per cent as compared with no deaths in control mice receiving identical intravenous doses but whose brains are left intact. It is essential that the time interval between injection of organisms and brain injury should not be too long and this is illustrated by the experiment recorded in Table 6. Mice were inoculated intravenously with broth culture and sterile starch-saline solution was injected intracerebrally at various time intervals afterwards. No deaths occurred if the starch injection was delayed for as long as 2 hours, by which time presumably many bacteria had been removed from the circulation.

TABLE 6

DEATHS IN MICE RECEIVING INTRAVENOUS DOSES OF *PAST. HAEMOLYTICA* FOLLOWED BY INTRACEREBRAL INJECTION OF STARCH SOLUTION AFTER VARIOUS INTERVALS

| *Dose of 6 hour broth culture intravenously* | *0·03 ml. of 2% starch solution intracerebrally* | *Interval between intravenous and intracerebral injections* | *Deaths in groups of 6 mice* |
|---|---|---|---|
| 0·4 ml. | + | <5 mins. | 6 |
| 0·4 ml. | + | 30 mins. | 3 |
| 0·4 ml. | + | 60 mins. | 2 |
| 0·4 ml. | + | 120 mins. | 0 |
| 0·4 ml. | — | — | 0 |

Strain of *Past. haemolytica* used was No. 7157H after 31 intracerebral mouse passages

*Intracerebral Protection Tests*

In the first of these experiments groups of mice received various dilutions of hyperimmune rabbit serum intraperitoneally. A further group received normal saline intraperitoneally in comparable volumes. Each mouse was subsequently challenged with *Past. haemolytica* injected intracerebrally. Deaths were recorded in each 24 hour period, and the results are shown in Table 7. It is obvious from the trivial differences between the final number of deaths in

each group that the effect of immune antibody on actual survival of mice in this experiment was slight if present at all. However, considering survivals on the second and third days it would again seem that 0·5 ml. of undiluted rabbit serum has lowered the resistance of mice to the infection and this is shown in the experiment as a more rapid death rate in these mice than in those receiving 1/5 serum. In the same way it will be seen that 1/5 hyperimmune rabbit serum tends to prolong survival time as compared with saline, but this protective effect is rapidly diluted to extinction. It is worthy of note that this experiment was performed on the same day as was the one which is recorded in Table 4, using the same batch of hyperimmune serum.

TABLE 7

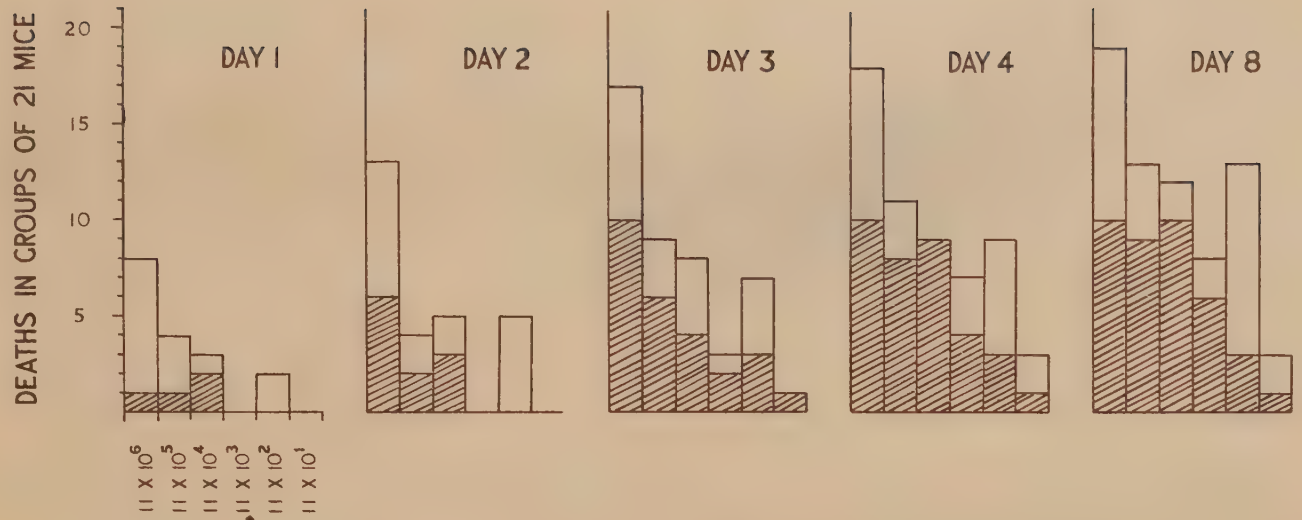
SURVIVAL TIMES IN MICE RECEIVING DILUTIONS OF HYPERIMMUNE RABBIT SERUM PRIOR TO INTRACEREBRAL CHALLENGE WITH *PAST. HAEMOLYTICA*

| | | *Showing the day after challenge on which each individual mouse died* | | | | | | | | | | | | | | | | | *Final deaths in groups of 18 mice* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dilution of immune serum | 1/1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 4 | 4 | 4 | 7 | S | S | 16 |
| | 1/5 | 1 | 1 | 2 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 10 | S | S | S | S | S | 13 |
| | 1/10 | 1 | 1 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | S | S | S | 15 |
| | 1/20 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 4 | 4 | 5 | S | S | S | 15 |
| Normal saline | | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | S | S | 16 |

Dose volume of serum dilutions and saline=0·5 ml. intraperitoneally.
Strain of *Past. haemolytica* used was No. 7157H after 42 intracerebral mouse passages.
Challenge dose per mouse=$0{\cdot}85 \times 10^6$ viable organisms.
S=Mouse survived.

Figure 1 illustrates the results of a protection test in which the effect of a constant dose of serum on graded challenge doses was studied. One hundred and twenty-six mice were inoculated intraperitoneally with 0·5 ml. of 1/5 hyperimmune rabbit serum, this dose being chosen from a consideration of the previous experiment. These mice were then divided into 6 equal groups which were subsequently challenged intracranially with ten-fold dilutions of organisms. One hundred and twenty-six control mice were treated in an exactly similar manner except that normal rabbit serum was substituted for hyperimmune. Deaths were recorded on each day after challenge. No deaths occurred after the 8th day. Although the protection afforded by immune serum against this method of challenge was not dramatic, a comparison of survivors among protected and control mice on each day did in fact demonstrate protection. The most striking comparison was obtained at the end of the first day.

Fig. 1




Effect of graded intracerebral doses of *Past. haemolytica* on mice pretreated with hyperimmune and normal rabbit serum.

Dose of appropriate serum per mouse 0·5 ml. of 1/5 dilution intraperitoneally.

Strain of *Past. haemolytica* used was No. 7157H after 42 intracerebral mouse passages.

Shaded areas represent total deaths to date in mice protected with immune serum. These are superimposed on clear areas which represent total deaths in control mice inoculated with normal serum.

Deaths in protected mice did not at any time or any challenge dose exceed deaths in control mice.

No deaths occurred after the 8th day.

## DISCUSSION

Mucin reduces by many millions the numbers of *Past. haemolytica* necessary to kill mice by intraperitoneal inoculation. This reduction represents a far greater number of bacteria than does the dose which, when combined with mucin, will produce a high mortality. These considerations are of importance in establishing true infections with bacteria such as *Past. haemolytica* which are normally of low virulence for laboratory animals and whose lethal properties may well be due in part to the toxicity of the necessarily large inoculum. The dose of certain strains of *Past. haemolytica* required to produce a high death rate is still comparatively large even though mucin is used but the results recorded in Table 1 suggest that this does not apply equally to all strains. It is, however, well illustrated by No. 7157II which has been used in all protection experiments. Nevertheless it is apparent that this method of challenge is likely to provide a satisfactory means of demonstrating and comparing protective potencies of various immune sera and of investigating cross antigenicity between strains in terms of immunising ability. Useful information on questions of immunity to *Past. haemolytica* might also be obtained if it should prove possible to produce active immunity in mice against this type of challenge.

Although the intracerebral method of challenge is capable of showing protection by hyperimmune rabbit serum, the demonstration of anything but gross differences in protective antibody content of sera by this means would not be possible without using large numbers of mice, and even this would probably be of no avail in attempted comparisons of sera of relatively low potency. Factors contributing to this state of affairs are the variation in susceptibility of mice to intracranial challenge, and the relative inefficiency of circulating rabbit antibody in combating cerebral infections with *Past. haemolytica* in mice. It must of course be borne in mind that rabbit antibody may be less efficient in this respect than that from other animal species.

Using intracerebral doses of organisms known from virulence titrations to be capable of producing high mortality, mice pretreated with hyperimmune serum have frequently shown increased survival times as compared with controls. This effect is seen especially over the first two or three days after challenge and is usually far more marked than actual protection from death. A number of factors may be responsible for this, including the high initial concentration of circulating antibody and also the trauma produced by the inoculating needle which probably brings about some temporary increase in permeability of the blood-brain barrier. A further increase in this permeability probably occurs at a later stage from the infective process, but by this time the blood level of antibody is undoubtedly much reduced.

## CONCLUSIONS

True infections of *Past. haemolytica* can be produced in mice by intraperitoneal inoculation of organisms mixed with mucin, or by intracerebral inoculation.

Using either method as a means of challenge it is possible to demonstrate protection in mice by hyperimmune rabbit serum injected intraperitoneally. For this purpose, however, intraperitoneal injection of organisms in mucin is by far the more successful of the two challenge procedures.

Comparatively large doses of rabbit serum administered intraperitoneally to mice influence the course of the disease produced by either method of infection, quite apart from the effects of any specific immune antibody.

A modified way of producing cerebral infections consists of the intravenous administration of organisms followed by injection of sterile starch solution into the brain.

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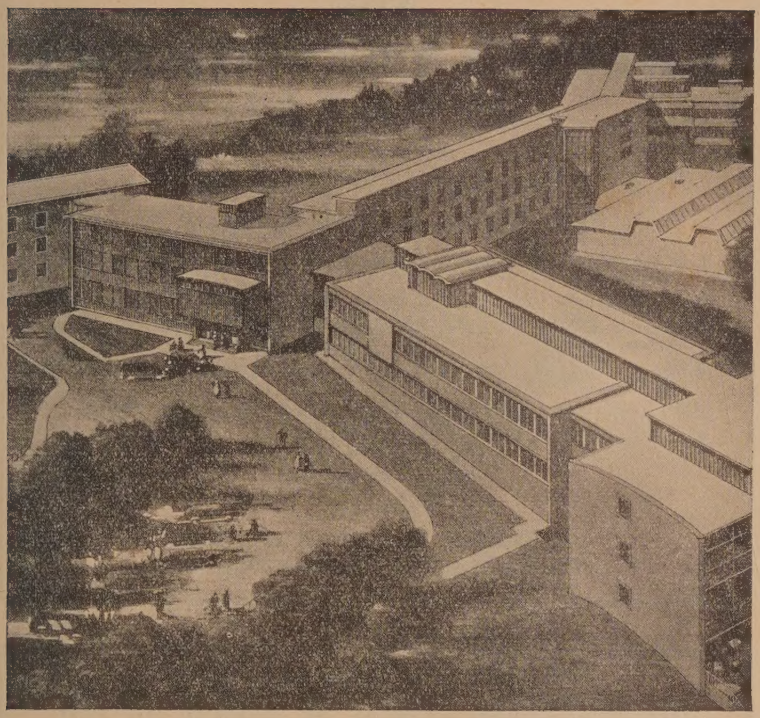
[*Received for publication, January 23rd,* 1958]



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